REMARKS

Claims 1-6, 8-23, 34-61 are pending in the application. Claims 24-33 have been canceled, herein. Claims 11, 13 and 34-56 have been withdrawn from consideration by the Examiner. Claims 8 and 14-18 have been amended. Claim 8 was amended to correct a typographical error. Claims 14-18 were amended to correct dependency of these claims. Claims 57-61 have been added. Claims 57-61 encompass the dependencies deleted from claims 14-18. No new matter has been added.

Claim Objections

The Examiner has objected to claims 14-18 under 35 CFR § 1.75(c) because a multiple dependent claim cannot serve as the basis for additional multiple dependent claims. Applicants have amended claims 14-17 so that they no longer depend from multiply dependent claims, and have amended claim 18 so that it no longer multiply depends. The dependencies deleted from claims 14-18 have been added in new claims 57-61. Applicants submit that this objection is overcome.

The Examiner has objected to claim 8 because of the phrase "abody fluid". This phrase has been corrected. Applicants submit that this objection is overcome.

Claim rejections

Rejections under 35 U.S.C. § 101

The Examiner has rejected claims 24-33 under 35 U.S.C. § 101 because they are not presented as proper process claims. Applicants have canceled claims 24-33. Thus this rejection has been rendered moot.

Rejections under 35 U.S.C. § 112

Indefiniteness

The Examiner has rejected claims 24-33 under 35 U.S.C. § 112 for being indefinite. Applicants have herein canceled claims 24-33. Thus this rejection has been rendered moot.

Enablement

The Examiner has rejected claims 1-6, 8-11, and 19-33 under 35 U.S.C. § 112 for lack of enablement for a method of diagnosing a pre-neoplastic state in a mammal. Applicants have herein canceled claims 24-33, so this rejection is moot as it regards these claims. The Examiner argued that while the specification does enable methods of staging or diagnosing cancer and methods of determining the etiology of carcinogenesis in a mammal,

that the specification does not contain teachings regarding issues involved in diagnosing pre-cancer of leukemia and solid tumors.

The Examiner argued that for the claim to be enabled for solid tumors there would have to be a pre-neoplastic stage at which expression would begin to deviate from the normal disclosed in the instant specification. The Examiner asserted that the instant specification does not teach such a deviation. Further, the Examiner stated that since chronic lymphocytic leukemia (CLL) in B cells is correlated with the t(11;14)(q13;q32) translocation, to detect the pre-cancer associated with CLL, the B cell would have to over-express $P2X_7$ receptor before the translocation took place.

Applicants submit that the specification teaches the detection of pre-cancer with antibodies to $P2X_7$.[1] The specification teaches on page 18, lines 8-18 that $P2X_7$ was upregulated in the prostatic epithelium of aged rats. The aged rats had a marked increase in epithelial hyperplasia resembling benign prostatic hyperplasia (BPH) in humans.[2]

Further the specification teaches differential staining of human prostate tissue in the process of early pre-neoplatic transformation.[3] The specification teaches three stages of P2X labeling associated with BPH tissue.[4] In stage 1, there is dense prominent P2X labeled epithelial nuclei on a pale background.[5] In stage 2, there is a de-expression of P2X in epithelial nuclei and the appearance of dense and punctuate cytoplasmic labeling, nuclear membrane and lateral plasma membrane labeling, and increase in labeling on the apical epithelium.[6] In stage 3, the nuclei were labeled with P2X only on the nuclear membrane, there was no cytoplasmic labeling, the labeling was homogenous instead of punctuate, and the apical epithelium was densely labeled.[7] Stages 1 and 2 of P2X labeling were usually found in histologically diagnosed normal tissue, while stages 2 and 3 of P2X labeling were usually found in histologically diagnosed cancerous tissue.[8] Stage 3 was always associated with histologically diagnosed cancerous tissue.[9] Thus, histologically normal tissue which was P2X labeled according to stage 2 would be considered pre-cancerous.[10] Thus,

---

[1] *E.g.* instant specification at 7, lines 1-3.
[2] *Id.* at 17, lines 16-18.
[3] *Id.* at 20, lines 5-9.
[4] *Id.* at 19, lines 16-17.
[5] *Id.* at 19, lines 17-19.
[6] *Id.* at 19, lines 19-22.
[7] *Id.* at 19, lines 22-25.
[8] *Id.* at 20, lines 1-2.
[9] *Id.* at 20, lines 3-5.
[10] *Id.* at 20, lines 6-9.

Applicants submit that the specification teaches the detection of pre-cancer with antibodies to $P2X_7$.

Moreover, later teachings in the art have confirmed that the presence of $P2X_7$ may be used to diagnose pre-cancer. The early appearance of $P2X_7$ in prostate epithelial cells in intracellular locations, including luminal cell nuclei, is observed with no associated deployment to the outer cell membrane.[11,12] These receptors which are not deployed are unable to function as non-selective fast-gated calcium channels or as apoptotic pores.[13] Also, it has been shown that $P2X_7$ receptors are expressed in ducts associated with ductal carcinoma of the breast in histologically normal tissue.[14] Moreover, apparently normal skin on the margin of a spreading melanoma stains for $P2X_7$ receptors, which presage the coming of cancer.[15]

While, as the Examiner stated in the Office Action, it is unclear whether $P2X_7$ may be used to diagnose pre-cancer of CLL, it is well recognized under U.S. law that even in unpredictable arts, a disclosure of every operable species is not required.[16] The MPEP states that detailed procedures for making and using the invention may not be necessary if the description of the invention itself is sufficient to permit those skilled in the art to make and use the invention.[17] The standard for determining whether the specification meets the enablement requirement is determining whether the experimentation needed to practice the invention is undue or unreasonable.[18] This standard has been interpreted to require that the claimed invention be enabled so that any person skilled in the art can make and use the invention without undue experimentation.[19] However, the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation.[20] As long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. § 112 is satisfied.[21]

Further, the quantity of experimentation needed to be performed by one skilled in the art is only one factor involved in determining whether "undue experimentation" is required to

---

[11] Slater, M. *et al.* Prostate Cancer and Prostatic Diseases 4:92-96 (2001), included as Exhibit A.
[12] Slater, M. *et al.* Histopathology 44:206-215 (2004), included as Exhibit B.
[13] *Id.*
[14] Slater, M., *et al.* Breast Cancer Research and Treatment 83:1-10 (2004), included as Exhibit C.
[15] Slater, M. *et al.* Melanoma Research 13:137-145 (2003), included as Exhibit D.
[16] In re Fisher, 427 F.2d. 833, 839 (Fed. Cir. 1970).
[17] M.P.E.P. § 2164.
[18] Mineral Separation v. Hyde, 242 U.S. 261, 270 (1916).
[19] In re Wands, 858 F.2d. 731, 737 (Fed. Cir. 1988).
[20] In re Wands, 858 F.2d. at 737.
[21] In re Fisher, 427 F.2d. at 839.

make and use the invention. An extended period of experimentation may not be undue if the skilled artisan is given sufficient direction or guidance.[22] The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.[23] Time and difficulty of experiments are not determinative if they are merely routine.[24]

The instant specification describes methods for using the expression of $P2X_7$ to diagnose pre-cancer. Specifically, Example 1 teaches the antibodies and immunohistochemical methods to be used. Example 2 teaches how the antibodies used in Example 1 were made and their specificity tested. Examples 4 and 5 teach how to prepare tissue for ultrastructural examination, and the immunocytochemistry involved in this analysis. Example 7 teaches the use of these above described methods to diagnose pre-cancer in rats. Example 9 teaches the use of the above described methods to diagnose pre-cancer in humans.

Applicants submit that the skilled artisan readily and routinely utilizes the described methods when histologically diagnosing cancer, only that, prior to the instant application, the skilled artisan would not have known to evaluate the expression profile of $P2X_7$. Further, although experimentation may be required to test the efficacy of $P2X_7$ to diagnose pre-cancer originating from the many different tissues or cancer types not disclosed, the instant specification provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed that the experimentation is not undue as the art typically engages in such experimentation. One of ordinary skill in the art would be able to practice the present invention as claimed as the specification discloses methods for making and using the claimed invention that bear a reasonable correlation to the entire scope of the claim. Thus, the enablement requirement is satisfied.

Rejections under 35 U.S.C. § 103

The Examiner has rejected claims 1-3, 8-10, 19-21 and 24-34 under 35 U.S.C. § 103 for being obvious over Altieri *et al.* WO 92/16558 ("Altieri") in light of Jamieson *et al.* Journal of Cellular Physiology, 166:637-642 (1996) ("Jamieson") and Buell *et al.* Blood, 92:3521-28 (1998) ("Buell"). Applicants have herein canceled claims 24-33, so this rejection is moot as it regards these claims. The Examiner asserted that claims 1 and 2, from which claims 3, 8-10, 19-21 and 24-34 ultimately depend are drawn to methods of staging or

[22] In re Colianni, 561 F.2d 220, 224 (CCPA 1977).
[23] In re Wands, 858 F.2d at 737.
[24] In re Wands, 858 F.2d at 737.

determining neoplastic states in a mammal or determining the etiology of neoplastic states in a mammal, by comparing the $P2X_7$ receptor expression profile of cells and/or tissues from said mammal to the $P2X_7$ expression profile of normal cells and/or tissues.[25] The Examiner asserted that Altieri teaches a method of diagnosing patients afflicted with chronic lymphocytic leukemia (CLL) by EPR-1 expression on cell surfaces correlated with an improvement of disease state. However, Altieri does not teach the use of $P2X_7$ as a marker for CLL. Additionally, the Examiner alleged that Jamieson teaches that lymphocytes from patients with CLL have higher levels of $P2X_7$ receptor. However, Jamieson does not teach the direct detection of $P2X_7$. Also, the Examiner asserted that Buell teaches a monoclonal antibody which binds to cells which express $P2X_7$ and which can be used for immunoprecipitations. Applicants traverse.

Applicants submit that the Examiner has not made a *prima facie* case of obviousness, and instead is using an "obvious to try" standard. "Obvious to try" is not the standard under § 103.[26] The MPEP defines what would have been "obvious to try" would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful.[27] Applicants submit that this is the standard necessary, specifically, in order to reach a conclusion, as explained below, that Jamieson teaches that $P2X_7$ expression on lymphocytes is increased in patients with CLL compared to expression on lymphocytes isolated from normal subjects. Applicants submit that Jamieson does not teach or suggest that $P2X_7$ is expressed at a higher (or lower) level in CLL lymphocytes than in normal lymphocytes.

Altieri teaches the monitoring of CLL via the monitoring of cell surface expression of EPR-1, which is homologous to coagulation factors V and VIII. There are no teachings that EPR-1 shares any significant homology with purinoceptors generally, or $P2X_7$, specifically. One of ordinary skill in the art would not expect $P2X_7$ expression to correlate with CLL disease state based on the teachings of Altieri.

Jamieson does not cure the deficiencies of Altieri. The Examiner alleged on the first full paragraph of page 6 of the Office Action that Jamieson teaches that lymphocytes from patients with CLL have higher levels of P2Z (*i.e.* $P2X_7$) receptors than lymphocytes from normal individuals at Figure 1 of Jamieson. Applicants disagree.

---

[25] Office Action at 5, paragraph 8.
[26] MPEP § 2145.
[27] *Id.*

Figure 1 does not explicitly show that the expression of $P2X_7$ is different in lymphocytes isolated from CLL patients and lymphocytes isolated from normal subjects. Figure 1 shows the increased loss of L-selectin in lymphocytes isolated from normal subjects and CLL patients which are treated with ATP. This reduced expression is shown as a percentage of L-selectin expression at time 0 of the ATP treatment. Jamieson does not teach what the absolute expression of L-selectin is in lymphocytes isolated from normal subjects or CLL patients, or what the relative expression of L-selectin is in lymphocytes isolated from normal subjects compared to CLL patients.

The Examiner seems to imply in the Office Action that because the relative percentage of L-selectin is lower in lymphocytes from CLL patients than it is in lymphocytes isolated from normal subjects[28] that one of ordinary skill in the art would reasonably conclude that the reason for this difference would be heightened expression of $P2X_7$ in the lymphocytes of the CLL patients. Jamieson teaches that purinoceptors like $P2X_7$ are involved in the increase of intracellular Ca^{2+} in lymphocytes in response to increases of extracellular ATP.[29] Jamieson also teaches that administration of ATP to CLL and normal lymphocytes results in rapid loss of L-selectin.[30] Jamieson shows that $P2X_7$ is involved in a signal transduction pathway which leads to the loss of L-selectin from lymphocytes by showing that the loss of L-selectin is not intracellular Ca^{2+} concentration dependent,[31] and that inhibition of the $P2X_7$ receptor stops L-selectin loss in lymphocytes,[32] while addition of benzoylbenzoic ATP, a more potent agonist of $P2X_7$ than ATP,[33] caused further increases of L-selectin loss.[34] However, Jamieson does not teach what the relative expression of $P2X_7$ is in CLL versus normal lymphocytes, nor does Jamieson teach what the relative or absolute expression of L-selectin is in CLL versus normal lymphocytes. Thus, it is even unclear whether the lymphocytes isolated from CLL patients even lose more L-selectin in absolute terms.

There are many explanations which could account for the results shown in Figure 1. One possibility is, as the Examiner seems to assert, that $P2X_7$ may be more plentiful on the cell surface of lymphocytes isolated from CLL patients than it is on the cell surface of lymphocytes isolated from normal subjects. However, there are many other possible

---

[28] Jamieson at 639, figure 1.
[29] *Id.* at 637, column 2.
[30] *Id.* at 638, column 1.
[31] *Id.* at 640, figure 5.
[32] *Id.* at 639, figure 3.
[33] *Id.* at 640, column 1.
[34] *Id.* at 640, figure 4.

explanations. Another possibility is that L-selectin levels are decreased in lymphocytes isolated from CLL patients than in lymphocytes isolated from normal subjects, leading to a greater percent decrease, but reflecting a similar absolute decrease in L-selectin. Another possibility is an increase in the activity of $P2X_7$ receptors found in lymphocytes isolated from CLL patients compared to receptors found in lymphocytes isolated from normal subjects. This could result in a greater decrease in L-selectin in the CLL lymphocytes as compared to normal lymphocytes, while $P2X_7$ expression levels remained the same. Another possibility is an increase in the expression or activity of another member of the signal cascade associated with the $P2X_7$ receptor in CLL lymphocytes. It is possible that, for example, an intracellular intermediate is the rate determining step between binding of ATP to the $P2X_7$ receptor and the loss of L-selectin from the lymphocyte surface. Another possibility could be the expression or activity of the protease, which is thought to be responsible for the cleavage of L-selectin from the cell surface of lymphocytes,[35] is increased in CLL lymphocytes as compared to normal lymphocytes. Thus, the same signal when transduced in CLL lymphocytes has a greater cleavage affect than the same signal in normal lymphocytes. Jamieson does not give guidance as to the probability of any of these possibilities, nor does it teach or suggest that $P2X_7$ is differentially expressed in CML versus normal lymphocytes.

The possible mechanisms for ATP's increased effect on the reduction of L-selectin on the surface of CLL lymphocytes, when compared to normal lymphocytes are many. The examples recited above are not exhaustive of all the possible mechanisms which are possible to explain the higher percentage loss of L-selectin in CLL lymphocytes as compared to normal lymphocytes.

Buell does not cure the deficiencies of Altieri and Jamieson. Buell merely teaches an antibody specific for $P2X_7$.[36] Buell does not even mention primary lymphocytes let alone differential expression of $P2X_7$ in CLL lymphocytes as compared to normal lymphocytes.

Thus, the rejection of claims 1-3, 8-10, 19-21 and 24-34 for obviousness over Altieri in light of Jamieson and Buell follow the "obvious to try" standard and do not make a *prima* facie case of obviousness. For one of ordinary skill in the art to ascertain the cause of differential percent L-selectin loss from CLL and normal lymphocytes from the combination of the teachings of Altieri, Jamieson, and Buell, he/she would have to try each of numerous possible choices to determine the mechanism of increased percent L-selectin loss in CLL lymphocytes treated with ATP compared to ATP treated lymphocytes from normal subjects

[35] *Id.* at 641, column 2.
[36] Buell at 3521, abstract.

until he/she possibly arrived at a successful result. None of Altieri, Jamieson, or Buell gave any indication of which parameters are critical and no direction as to which of many possible choices is likely to be successful. Thus, the combination of Altieri, Jamieson, and Buell do not make a *prima facie* case of obviousness for claims 1-3, 8-10, 19-21 and 24-34 and Applicants request that this rejection be withdrawn.

The Examiner has also rejected claims 1-3, 8-10 and 19-34 under 35 U.S.C. § 103 for being obvious over Altieri in light of Jamieson, Buell and Rio *et al.* WO 97/06256 ("Rio"). Applicants have herein canceled claims 24-33, so this rejection is moot as it regards these claims. The Examiner asserted that claim 22 embodies claims 1 and 2 where the means of detection is a Western blot.[37] Altieri, Jamieson and Buell do not teach or suggest a Western blot. The Examiner asserted that Rio teaches detection of leukemia markers by Western blot.[38] Applicants traverse this rejection.

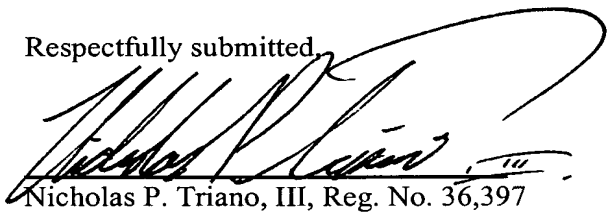
While Rio may teach Western blotting, its teachings do not cure the deficiencies of the teachings of Altieri, Jamieson and Buell described above. Rio does not teach the differential expression of $P2X_7$ in cancer or pre-cancer cells or tissues versus normal cells or tissues, nor does it indicate the cause of differential percent L-selectin loss in CLL lymphocytes as compared to normal lymphocytes shown in Figure 1 of Jamieson. Thus, for this reason, and the reasons asserted above, Applicants submit that there is no *prima facie* case of obviousness of claims 1-3, 8-10 and 19-34 in light of Altieri over Jamieson, Buell and Rio, and request that this rejection be withdrawn.

[37] Office Action at 6, paragraph 9.
[38] *Id.* at 7, first paragraph.

CONCLUSION

Applicants submit that the claims as here amended put the application in condition for allowance, and such action is respectfully requested. Should any questions or issues arise concerning the application, the Examiner is invited and encouraged to contact the undersigned at the telephone number provided below.

Respectfully submitted,



Nicholas P. Triano, III, Reg. No. 36,397
Attorney for Applicants
MINTZ, LEVIN, COHN, FERRIS
GLOVSKY and POPEO, P.C.
Tel: (617) 542-6000
Fax: (617) 542-2241
Customer No. 30623

Dated: December 19, 2005

TRA 2104477v.1

Exhibit A

Prostate Cancer and Prostatic Diseases (2001) 4, 92–96
 1365–7852/01 $15.00
www.nature.com/pcan

Detection of preneoplasia in histologically normal prostate biopsies

MD Slater[1]*, WJ Delprado[2], CR Murphy[1] & JA Barden[1]

[1]*Institute for Biomedical Research, The University of Sydney, Sydney, Australia; and*
[2]*Douglass Hanly Moir Pathology, North Ryde, Australia*

P2X immunolabeling of prostate detected preneoplastic changes in apparently normal tissue. Labeling occurred in two well-defined stages before the diagnostic histological markers of cancer were visible. As cancer progressed, the location of P2X expression changed from confinement within individual nuclei in the acini (stage 1) to a cytoplasmic punctate label in the acinal epithelium, with an associated removal of nuclear stain (stage 2). Finally, in advanced cases, where clear morphological evidence of cancer was apparent, the P2X label condensed exclusively on the apical epithelium (stage 3). BPH/normal tissue was entirely devoid of P2X label. Biopsy samples (77) were tested in three categories. One group (35) were diagnosed as normal benign prostatic hyperplasia (BPH) on the basis of haematoxylin and eosin (H&E) stain, although underlying disease was suspected. Of these, 14 (40%) were clearly normal and appeared entirely devoid of label, 13 (37%) exhibited the first stage of P2X receptor labeling and the remaining eight (23%) exhibited second stage labeling. The accompanying H&E-stained sections of all these cases had a normal appearance. Low grade cancer biopsy samples with Gleason scores G4–7 (25) all revealed widespread second stage receptor labeling in areas of both normal and cancerous morphology, while 17 high grade cancer biopsy samples (Gleason G8–10) all showed third stage labeling along with some residual second stage labeling. The features of each P2X labeling stage occupied the entire histological area affected, offering more opportunity to diagnose the tissue than was supplied by the more-localised diagnostic features identified by H&E-stain. Besides detecting cases of preneoplasia in biopsies with a normal H&E appearance, this technique was also able to rule out the presence of neoplasia in purely hyperplasic prostates by the absence of any P2X labeling. *Prostate Cancer and Prostatic Diseases* (2001) 4, 92–96.

Keywords: prostate cancer; diagnosis; P2X receptor; calcium channels

Introduction

The autonomic nervous system plays a major role in the homeostatic, growth and secretory functions of the prostate.[1] In vertebrates, the autonomic P2X receptors/channels of the purinergic nervous system constitute a newly-described class of fast response, ligand-gated, calcium-permeable and cation-selective ion channels activated by extracellular ATP from nerve terminals or a local tissue source.[2] The P2X receptors are widely-distributed in mammalian tissues.[3] P2X receptor binding on the cell membrane triggers an influx of cytosolic Ca^{2+} into the cytoplasm, producing ionic concentrations that vary in different cellular compartments.[4] The intracellular Ca^{2+} signal is transduced via more than 40 Ca^{2+}-binding proteins including the S100 proteins, tenascin, calmodulin, integrin and annexin.[5] These calcium-bound proteins activate and regulate the cell cycle, protein secretion, the composition of nuclear proteins, DNA transcription, apoptosis, adhesion protein binding, cell differentiation and phosphorylation.[6] Changes in cytosolic Ca^{2+} therefore control a wide range of cellular responses.

Purines and pyrimidines can either stimulate or inhibit proliferation depending on the extracellular microenvironment, the physiological state of the target cells, cell cycle and the expression of P2X receptors.[7] Several lines of evidence suggest that P2X receptor expression, and the

*Correspondence: MD Slater, Institute for Biomedical Research, Department of Anatomy and Histology, The University of Sydney, Sydney, NSW 2006, Australia.
Received 3 July 2000, accepted 26 October 2000

resulting variations in Ca^{2+} homeostasis, may contribute to the progression of prostate cancer.[7-10] Extracellular Ca^{2+} influx also activates invasion and malignancy-associated enzyme metalloproteinases.[11] As an extension of studies on the role of P2X receptors in the urogenital system[12-16] we examined their expression in clinical biopsies that had been previously diagnosed, using a unique suite of subtype-specific antibodies.

Methods

Antibodies against unique epitopes in the extracellular domains of each P2X subtype were developed and their specificity determined using cDNA expressed in *Xenopus* oocytes and HEK293 cells. Each antibody exhibited specific labeling in rat, mouse and human tissue.[12-16] After optimisation and standardisation of P2X subtype antibody production and labeling characteristics, we evolved a standardised protocol. $P2X_1$ and $P2X_2$ had similar distributions. $P2X_3$ and $P2X_7$ labeled the same structures but obscured the clarity of the epithelial label because they exhibited denser staining of the prostatic nerve supply in the stroma. $P2X_4$, $P2X_5$ and $P2X_6$ did not label any components of normal or neoplastic tissue. Accordingly, we chose a mixture of affinity-purified $P2X_1$ and $P2X_2$ subtypes, each at a concentration of 0.5 µg/mL IgG in PBS. This $P2X_{1,2}$ mixture resulted in increased clarity of the features described, without an increase in stromal labeling.

Sections cut from blocks from cases that had been previously diagnosed were supplied by private clinicians and hospital pathology departments, as 5–10 µm paraffin-embedded sections of prostate biopsy tissue on glass slides. The mean age of the patients was 72 ± 9 y. Of these cases, 35 had previously been diagnosed as normal and/or containing BPH. All these cases contained some degree of epithelial hyperplasia. Twenty-five cases were in the Gleason score range 4–7, while 17 were Gleason 8–10. Those that had Gleason scores in the range 4–10 all had PSAs over 8 and/or rising. In no case previously diagnosed as normal/BPH, were prominent nucleoli observed. However, in 66% of cases with a Gleason score of 6 or more, prominent nucleoli were observed.

The supplied cases were de-waxed in two changes of fresh Histoclear for 10 minutes each and rehydrated. Sections were incubated for 30 min in 3% hydrogen peroxide in 1% bovine serum albumin in phosphate buffered saline (PBS), and washed in PBS 3 × 5 min. Sections were then incubated with an equal mixture of $P2X_{1,2}$ subtypes at 0.5 µg/mL IgG in PBS, for 30 min. Thereafter, slides were washed three times in PBS for 10 min each, followed by a 30 min incubation with a 1:100 dilution of HRP-conjugated goat anti-rabbit secondary antibody (Dako, Carpentera, CA). All slides were then washed three times in PBS for
10 minutes each, visualised using a 0.05% solution of diamino benzidine (DAB) for 10 min, washed, dried and mounted in Entellin mounting medium (Merck, Darmstadt, Germany). Approximately serial sections were stained with a standard H&E protocol and labeled with anti-P2X antibodies as described, respectively. For practical, ethical and, legal reasons, only formalin-fixed, paraffin-embedded and previously diagnosed biopsy samples were used. Conjugate epitopes completely blocked labeling in pre-absorption controls.

Results

Previously-diagnosed prostate cancer clinical specimens were used to identify three distinctly separate patterns of purinergic receptor (P2X) expression with increasing grade of cancer. Of the P2X subtypes ($P2X_{1-7}$), a mixture of $P2X_{1,2}$ antibodies resulted in optimal labeling patterns. In normal (non-neoplastic) tissue containing varying degrees of benign prostatic hyperplasia (BPH), no $P2X_{1,2}$ label (or any other subtype) was present. In samples clinically diagnosed as high grade cancer (Gleason grade G8–10), a distinctive labeling pattern was routinely observed in different regions. In early stage cancer (Gleason grade G4–7) stage 1 and 2 P2X labeling patterns predominated, although stage 3 labeling was observed in advanced cancer, particularly G7 tissue. These three distinct patterns were designated as purinergic receptor translocation stages 1–3 (PRT 1–3). PRT 1 involved intense labeling of prostatic epithelial nuclei while in PRT 2, punctal cytoplasmic P2X receptor translocation predominated, with the (previously labeled) nuclei now unlabeled. All label within the nuclei had migrated outside the nuclei. PRT 3 involved intense P2X receptor labeling on the apical epithelium in those cells possessing clear morphological changes. In the most advanced cases of cancer only PRT 3 was observed. However, in regions of tissue from cases of advanced cancer where the cellular morphology was merely hyperplasic or appeared entirely normal, PRT 2 and very occasionally PRT 1 were observed.

Patient diagnoses and case notes were used as a guide to separate the cases of normal/BPH (taken from patients obviously suspected of harboring cancer) from early stage and from late stage cancer. Staining patterns in TURP chips and biopsy core specimens were identical. In normal and/or BPH tissue, as originally diagnosed by H&E staining as well as long-term patient outcome, P2X labeling was totally absent.

Figure 1a is an H&E-stained section of a core biopsy sample taken from a 71-y-old man diagnosed with BPH while Figure 1b shows an anti-P2X immunoperoxidase-labeled (approximately) serial section showing the complete lack of P2X receptor expression characteristic of non-neoplastic BPH tissue. This lack of staining was seen in 40% (14 of 35) of cases diagnosed as normal/BPH by H&E. Of the 14 normal, 12 had normal PSA levels, while in two cases the PSA levels were slightly increased. We propose that these cases represent genuinely normal tissue, with no evidence of any preneoplastic change.

The earliest stage of P2X labeling (PRT 1) was characterised by dense, prominently-labeled epithelial nuclei (PEN) surrounded by an unlabeled stroma. Dilution studies revealed this pattern was composed of a dense accumulation of P2X receptor/channel puncta in the nucleus, that were entirely absent in genuinely normal/BPH tissue. PRT 1 labeling was seen in 60% (21 of 35) of biopsied samples suspected of harboring some cancer but

diagnosed as normal/BPH on the basis of H&E staining. A total of 16 of these 21 cases had normal PSA levels while the other five showed a slight increase. Figure 2a is a micrograph of an H&E-stained core biopsy from a 56-y-old patient with a diagnosis of BPH with 'slowly increasing' PSA but with no cancer identified. The acini appear to be entirely normal. Figure 2b is a micrograph of the same group of acini, in an (approximately) serial section, immunolabeled with anti-P2X antibodies showing the characteristic prominent epithelial nuclei (PEN-arrow) of PRT 1 despite its benign H&E appearance.

The second stage (PRT 2) was characterized by the appearance of P2X receptor puncta throughout the cytoplasm. This transport of receptors resulted in progressive de-expression of the (PRT 1) nuclear label to an end-point where the nuclei were completely devoid of label. These nuclei appeared outlined by the presence of cytoplasmic label. Some residual label occasionally could be found on the nuclear membrane itself. Figure 3a shows a H&E-stained core biopsy from a 57-y-old man with an increasing PSA. The clinical diagnosis was (mild) BPH with an adjacent area of cancer diagnosed as Gleason score 6. The area depicted in Figure 3a is from the area diagnosed as BPH and devoid of cancer. Figure 3b is an anti-P2X immunoperoxidase-labeled (approximately) serial section showing the same acinal cluster as Figure 3a. PRT 2 is readily apparent. In some regions of tissue diagnosed as advanced cancer, PRT 2 was sometimes observed although stage PRT 3 labeling was far more common. Figure 4a shows a core biopsy from a 69-y-old patient with increasing PSA. The clinical diagnosis was Gleason score 9. All acini were abnormal by H&E stain. Figure 4b is a micrograph of an anti-P2X immunoperoxidase-labeled (approximately) serial section showing the same acinal cluster as Figure 4a. PRT 2 is readily apparent (arrow), appearing as a distinctive, punctate cytoplasmic-translocating receptor label.

The final stage (PRT 3) consisted of an apical deposition (AD) of intensely labeled receptors on the apical epithelium. The nuclei remained devoid of label but the translocating puncta in the cytoplasm disappeared as the receptors deposited on the apical epithelium. The AD label was characteristically dense, homogeneous rather than punctate and relatively thick—a feature probably intensified by the contribution of apical labeling in the section below the plane of focus. In cases where the histological appearance by H&E stain was obviously cancer, PRT 3 was predominent. Figure 5a is a core

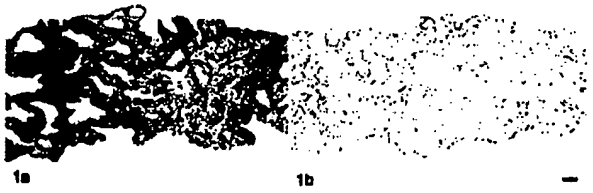


Figure 1 *Panel (a) shows a H&E stained section of biopsy core sample from a 71-y-old man with increasing PSA. The clinical diagnosis was BPH. Figure 1(b) is an immunolabeled (approximately) serial section demonstrating non-expression of P2X receptors in BPH/normal tissue. Bar = 50 microns.*

Figure 2 *Panel (a) shows a H&E stained core biopsy specimen from a 56-y-old with 'slightly increasing' PSA. The clinical diagnosis was BPH. Figure 2(b) is an immunolabeled (approximately) serial section demonstrating PRT 1 (prominent epithelial nuclei or PEN—arrow), suggesting the onset of preneoplasia. Bar = 20 microns.*

Figure 3 *Panel (a) shows a H&E stained core biopsy specimen from a 57-y-old with 'increasing' PSA. The clinical diagnosis was BPH in the area depicted with an adjacent area of Gleason score 6. Figure 3(b) is an immunolabeled (approximately) serial section demonstrating PRT 2 indicating the likely presence of cancer within the tissue or an advanced case of preneoplasia suggesting the imminent onset of cancer. Bar = 20 microns.*

biopsy taken from an 86-y-old patient with increasing PSA. The clinical diagnosis was Gleason score 7. All acini in the biopsy were abnormal by H&E stain and contained numerous prominent nucleoli (arrow). Figure 5b shows an anti-P2X immunoperoxidase-labeled (approximately) serial section of the same acinal cluster as Figure 5a. PRT 3 predominates, as characterised by the intense deposition of receptors on the apical epithelium (arrow).

Discussion

This study describes three stages in progressive prostate cancer consisting of P2X receptor/channel translocation from the nucleus to the plasma membrane via the cytoplasm. All biopsy specimens were taken from patients who presented with symptoms of prostate disease. The presence of PRT 1 or PRT 2 labeling features in tissue diagnosed as normal/BPH suggests that the neoplastic process begins at a metabolic level, some time prior to the appearance of H&E-stained diagnostic features. It is unlikely that these labeled receptors are acting as functional calcium channels in the nucleus at this early stage of expression and prior to being transported to epithelial cell surfaces.[17]

The observation of PRT 1 and PRT 2 in tissue diagnosed as normal by H&E staining is suggestive of preneoplasia given the presence of PRT 2–3 in cases of obvious cancer in which morphological changes are observed. Of the 35 cases previously diagnosed as normal/BPH, 40% (14 of 35) exhibited no P2X expression at all. This observation suggests that these tissues did not harbor any preneoplasia. A further 37% (13 of 35) contained PRT 1, while 23% (8 of 35) contained PRT 2. A finding of PRT 2 features in tissue diagnosed as normal was suggestive of comparatively aggressive or at least more advanced preneoplasia. No normal/BPH cases contained features of PRT 3, as this stage was always accompained by obvious carcinogenic changes to the cellular morphology. In each of the 25 cases previously categorised as Gleason score G4–7, PRT 2 was observed with some PRT 1 but not PRT 3. In the 17 cases previously diagnosed in the range G8–10, PRT 3 was always seen, with occasional or no features of PRT 2. In biopsy tissue that was well removed from a site of diagnosed cancer and that was normal by H&E staining, PRT 2 labeling and even some areas of PRT 1 labeling was noted. The PRT staining pattern was exclusive to the entire histological area of a particular stage. Often only a single core showed any cancer with all other cores appearing normal or BPH by H&E but all these cores contained clear signs of PRT 1 and/or PRT 2. We propose that all the cores that showed these features of PRT 1 and PRT 2 indicated the presence of underlying transformational change.

An explanation for our findings may be found in the unique metabolism of prostate cells. In cells from any other body tissue, citrate provides fuel for oxidation in the Krebs cycle and the production of ATP. The unique prostate cell Krebs cycle works in a fundamentally different manner with citrate being an end-product of oxidation rather than the fuel for oxidation.[18–20] Glucose and pyruvate are then used as substitutes for citrate. This results in an extremely inefficient energy metabolism and a 10–30 times greater citrate concentration in the prostatic cytosol than is found in all other cells of the body. Citrate and zinc, essential for the proper functioning of the sperm, are ultimately secreted into the seminal plasma where the Zn^{2+} binds to citric acid and glycoproteins. Prostatic citrate production is regulated by testosterone and prolactin. The mechanisms of this process have not been elucidated, but are generally believed to involve

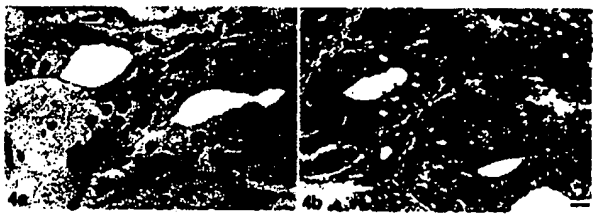


Figure 4 *Panel (a) shows a high power detail of a core biopsy specimen from a 69-y-old with increasing PSA. Clinical diagnosis is Gleason score 9 with all acini appearing abnormal by H&E. Figure 4(b) is an immunolabeled (approximately) serial section that clearly shows the translocating receptors (arrow) in PRT 2. Most areas had already progressed to PRT 3. Bar = 20 microns.*

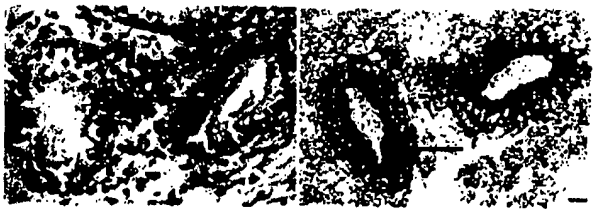


Figure 5 *Panel (a) shows high power detail of a core biopsy sample from a 86-y-old with increasing PSA. Clinical diagnosis is Gleason score 7. All acini were abnormal by H&E stain and contained numerous prominent nucleoli (arrow). Figure 5(b) is an immunolabeled (approximately) serial section showing the same acinal clusters. PRT 3 predominates, as shown by intense concentration of receptors on apical epithelium (arrow). Bar = 20 microns.*

tyrosine kinase-mediated pathways. Both zinc and citrate are significantly increased in BPH tissue (due to the increased numbers of secretory cells) and markedly decreased in prostate cancer. Normal prostate epithelial cell citrate levels are typically 4000–6000 nmol/g. In the proliferating cells of BPH tissue they are extremely high (8000–12 000 nmol/g) but very low in cancer cells (1000–2000 nmol/g).[21] This results from the reversion of cancer cells to the normal somatic Krebs cycle. The neoplastic transformation of prostate cells therefore involves radical metabolic change. It is probable that the varying P2X Ca^{2+} channel expression observed in the current study is the direct result of this altering metabolism. The phenomenon of calcium-associated receptor translocation in cancer is not unique. A similar translocation sequence to that described in this study has been reported for the malignancy-associated, calcium-binding S100 proteins in smooth muscle cells following intracellular Ca^{2+} influx.[22] An antibody to this protein family, anti-human S100B, has been used as a test for malignant melanoma for some time. Moreover, keratin expression studies have shown that some BPH tissue possesses an intermediate phenotype which may have premalignant potential suggesting that BPH and cancer may be etiologically related.[23] Our results clearly support this suggestion.

We propose that the use of purinergic receptor translocation (PRT) staging constitutes an effective tool with which to readily differentiate patients with non-cancerous BPH from those with early cancer, as yet undetectable by H&E staining, in men who present with elevated PSA levels and/or enlarged prostates. These features are clearly stage-specific, appear in each lobe of the prostate and extend a large distance from sites of cancer as diagnosed by H&E stain. This feature reduces the need to take many needle biopsy samples in the hope of sampling a diagnostic group of cells, currently required for H&E diagnosis. Data obtained by P2X immunolabeling appears to be unrelated to that of H&E staining, as it flags the metabolic changes that accompany neoplasia before the appearance of morphological change.

Regardless of whether the observed pattern anticipates the development of cancer, or is a consequence of a field effect in which the cancer process influences the adjacent epithelium,[23] the result remains that the presence of cancer can be detected indirectly and truly negative cases can be readily distinguished. This method may also provide data on the rate of progression of individual tumours.

References

1 McVary KT, McKenna KE, Lee C. Prostate innervation. *Prostate* 1998; 8(Suppl): 2–13.
2 Barnard EA, Simon J, Webb TE. Nucleotide receptors in the nervous system. An abundant component using diverse transduction mechanisms. *Mol Neurobiol* 1997; 15: 103–129.
3 Abbracchio MP, Burnstock G. Purinergic signalling: pathophysiological roles. *Japanese J Pharmacol* 1998; 78: 113–145.
4 Llinas R, Sugmori M, Silver R. The concept of calcium concentration microdomains in synaptic transmission. *Neuropharmacology* 1995; 34: 1443–1451.
5 Heizmann CW, Cox JA. New perspectives on S100 proteins: a multi-functional Ca^{2+}, Zn^{2+} and Cu^{2+} binding protein family [Review]. *Biometals* 1998; 11: 383–397.
6 Keirsebilck A *et al*. E-Cadherin and metastasin (Mts-1/S100a4) expression levels are inversely regulated in two tumor cell families. *Cancer Res* 1998; 58: 4587–4591.
7 Gong Y *et al*. Calcium regulation of androgen receptor expression in the human prostate cancer cell line LNCaP. *Endocrinology* 1995; 136: 2172–2178.
8 Lelievre V, Muller JM, Falcon J. Adenosine modulates cell proliferation in human colonic adenocarcinoma. I. Possible involvement of adenosine A1 receptor subtypes in HT29 cells. *Eur J Pharmacol* 1998; 341: 289–297.
9 Wasilenko WJ *et al*. Calcium signaling in prostate cancer cells: evidence for multiple receptors and enhanced sensitivity to bombesin/GRP. *Prostate* 1997; 30: 167–173.
10 Slater M, Murphy CR. Detection of apoptotic DNA damage in prostate hyperplasia using tyramide-amplified avidin-HRP. *Histochem J* 1999; 31: 747–749.
11 Dethlefsen SM *et al*. Extracellular calcium influx stimulates metalloproteinase cleavage and secretion of heparin-binding EGF-like growth factor independently of protein kinase C. *J Cell Biochem* 1998; 69: 143–153.
12 Hansen MA *et al*. Structural motif and characteristics of the extracellular domain of P2X receptors. *Biochem Biophys Res Comm* 1997; 236: 670–675.
13 Hansen MA *et al*. The distribution of single $P2X_1$ receptor clusters on smooth muscle in relation to nerve varicosities in the rat urinary bladder. *J Neurocytol* 1998; 27: 529–539.
14 Hansen MA *et al*. P2X (purinergic) receptor distributions in rat blood vessels. *J Autonomic Nervous System* 1999; 75: 147–155.
15 Dutton JL *et al*. Development of P2X receptor clusters on smooth muscle cells in relation to nerve varicosities in the rat urinary bladder. *J Neurocytol* 1999; 28: 3–15.
16 Worthington RA *et al*. Localisation of P2X receptors in HSG and HEK cells by SDS-PAGE/Western blotting and immunofluorescence. *Electrophoresis* 1999; 20: 2065–2070.
17 Dutton JL *et al*. P2X1 receptor membrane distribution and down-regulation visualized using receptor-coupled green fluorescent protein. *Neuropharmacology* 2000; 39: 2054–2066.
18 Costello L, Franklin R. Citrate metabolism of normal and malignant prostate epithelial cells. *Urology* 1997; 50: 3–12.
19 Costello L, Franklin R. Concepts of citrate production and secretion by prostate: 1. metabolic relationships. *Prostate*. 1991; 18: 25–46.
20 Liu Y, Franklin RB, Costello LC. Prolactin and testosterone regulation of mitochondrial zinc in prostate epithelial cells. *Prostate* 1997; 30: 26–32.
21 Franklin R, Costello L. *Intermediary Energy and Metabolism of Normal and Malignant Prostate Epithelial Cells*. CRC Press: New York, 1997, 115–150.
22 Mandinova A *et al*. Distinct subcellular localization of calcium binding S100 proteins in human smooth muscle cells and their relocation in response to rises in intracellular calcium. *J Cell Sci* 1998; 111: 2043–2054.
23 Xue Y *et al*. Changes in keratin expression during the development of benign prostatic hyperplasia. *Eur Urol* 1997; 32: 332–338.

Exhibit B

Histopathology 2004, 44, 206–215

Early prostate cancer detected using expression of non-functional cytolytic $P2X_7$ receptors

M Slater, S Danieletto,[1] A Gidley-Baird, L C Teh & J A Barden
Biosceptre Research Laboratory, Institute for Biomedical Research and Department of Anatomy and Histology F13, The University of Sydney, Sydney, and [1]Department of Pathology, Sydney Adventist Hospital, Wahroonga, Australia

Date of submission 6 March 2003
Accepted for publication 5 June 2003

Slater M, Danieletto S, Gidley-Baird A, Teh L C & Barden J A
(2004) *Histopathology* 44, 206–215

Early prostate cancer detected using expression of non-functional cytolytic $P2X_7$ receptors

Aims: To detect early prostate cancer reliably by monitoring the expression of non-functional $P2X_7$ cytolytic purinergic receptors.
Methods and results: $P2X_7$ receptors were absent from normal prostate epithelium obtained from post mortem tissue and tissue from cases of transurethral resection collected from young men ($n = 23$) who were confirmed to be free of cancer at later procedures 5–10 years after collection of the original samples. However, $P2X_7$ was present in every case of 116 confirmed prostate cancers regardless of Gleason grade or patient age. $P2X_7$ was present in apparently normal epithelial cells in acini well outside the tumour margins, but appeared in a distinct stage-specific manner commencing with the nucleus, progressing to the cytoplasm and collecting finally on the apical membrane of the epithelial cells in morphologically distinct cancer. The pattern of $P2X_7$ receptor localization in the epithelial cells was recorded in earlier biopsies obtained from the same patient cohort. One hundred and fourteen of 116 prostates stained positively for $P2X_7$ at the earliest biopsy, though generally with a less advanced pattern of distribution.
Conclusions: The appearance of $P2X_7$ receptors in normal prostate tissue adjacent to prostate tumours makes direct tumour biopsy less critical for positive cancer diagnosis and enables cancer progression to be monitored.

Keywords: prostate cancer, diagnostic, $P2X_7$ calcium channel receptor, apoptosis, immunolabelling

Abbreviations: PIN, prostatic intraepithelial neoplasia; PSA, prostate specific antigen

Introduction

Prostate cancer is the second commonest cause of cancer death in males after lung cancer in Western society. Although the incidence of prostate cancer is increasing in many countries, there is a lack of reliable prognostic indicators able to predict the behaviour of individual tumours. Latent prostate cancer and the preinvasive form of neoplasia known as prostatic intraepithelial neoplasia (PIN) have been found in 45% of autopsy specimens following death from trauma from men in their 50s to 82% of men aged in their 80s.[1] In Australia, a total of 12 500 new cases of prostate cancer were detected in the year 2000, of which 8000 were already well advanced with medium- to high-grade Gleason score and with many (20–30%) metastatic, a similarly high rate to other Western countries.[2] The advanced stage of the disease at initial diagnosis often results from the difficulty of detecting tumours and to cases in which the onset of disease is rapid. Thus, more effective early detection of clinically significant cancers is a high priority, particularly among those men who present with elevated prostate specific antigen (PSA) and who appear negative for cancer at initial biopsy. In the same way that $P2X_7$ receptor detection has been shown to differentiate between cases of aggressive and more benign breast cancers[3] both lobular and ductal, it is hoped that a

Address for correspondence: Dr Julian A Barden, Biosceptre Research Laboratories, Institute for Biomedical Research and Department of Anatomy and Histology F13, The University of Sydney, Sydney NSW 2006, Australia. e-mail: julian@anatomy.usyd.edu.au

similar detection system will prove useful in prostate cancer. In addition, the $P2X_7$ might be valuable in indicating which patients with high-grade PIN have concomitant cancer.

The purinergic (P2X) receptors contribute significantly to Ca^{2+} influx to the cytoplasm, regulating at least 40 intracellular calcium-binding proteins. These control protein secretion, cell motility and adhesion, invasiveness, cytoskeletal modification, cell junction assembly, tissue differentiation, and phosphorylation as well as nuclear matrix protein composition and expression, cell cycle regulation and DNA transcription.[4] The ionotropic subtypes $P2X_1$ and $P2X_2$ are expressed in prostate epithelium when tumours are found, but not in normal tissue.[5,6] These changes occur simultaneously with the appearance of telomerase-associated protein.[6] The appearance of low levels of telomerase activity in apparently normal tissue adjacent to tumours, including lung, skin, gut, bladder, pancreas, kidney, cervix, vulva and prostate, is suggestive of a field-effect of biochemical changes associated with a biochemical transformation that is undetectable with the use of common histological stains.[7–9] It is unsurprising that several of the P2X receptors alter their expression in the acinar epithelial cells in the prostate in advance of the morphological changes associated with the development of prostate cancer given the major biochemical changes that occur in tissue undergoing neoplastic transformation.

The class of purinergic receptors of type P2X is comprised of fast, ligand-gated cation channels that open in response to the binding of extracellular ATP. The seven subtypes designated $P2X_{1-7}$ exhibit extensive homology (30–40%), particularly in structurally important elements such as the extracellular disulphide bridges. The long intracellular C-terminal domain unique to $P2X_7$ appears to confer time-dependent dilatation of the channel into a pore that possesses the capability of transporting large organic cations with molecular weights of up to several hundred Daltons that initiate apoptosis in the cell.[10–12]

Our aim has been to detect the early onset of prostate cancer in patients by identifying tissue either in the process of becoming neoplastic or that may be influenced by a tumour developing in adjacent prostatic tissue. A further aim has been to detect reliably the presence of existing, usually small low–moderate-grade tumours that may have escaped detection at biopsy sampling through the effects of the tumour on adjacent and morphologically normal prostatic epithelium and which are likely to become clinically significant.

Materials and methods

PROTOCOL DESIGN

Needle biopsies were tested from 116 men (age range 47–86 years) who had been biopsied more than once to detect the presence of prostate cancer. Twenty of the patients were biopsied three to four times, the remainder twice. Prostate samples, either needle biopsies or tissue obtained from transurethral resection, were also collected from 17 men (age 38–59 years) who had presented with symptoms of benign prostate hyperplasia between 5 and 10 years before confirmation that they remained free of prostate cancer. The earlier samples were tested from these men and six post mortem tissues were obtained from men aged 20–25 years for control purposes. These were fully sectioned at intervals to exclude cancer. No positive label was seen in associated tissue epithelium including urothelium. All samples were otherwise randomly selected. The 116 confirmed cancer cases were in the range Gleason sum score 4–9. Most of these patients were considered to be false negatives at the initial biopsy with tumours that were present but which were missed by the biopsy needles and were thus kept under close observation. They were consequently re-biopsied between 3 months and 6 years (up to three times) after the initial biopsies and were then all confirmed with cancer at a final biopsy when the tumour was directly sampled. Indications for re-biopsy included continuing high or increasing PSA levels and/or continued clinical suspicion based on ultrasound testing. Over half the cases were detected within 15 months of the initial biopsy. Sections (4–5 μm) were cut from an average of eight cores taken from the peripheral zone per procedure but ranging from three to 12 (apex, mid, base from left and right) for each sample.

TISSUE LABELLING

Each slide was de-waxed in two changes of fresh Histoclear for 10 min and then rehydrated. Sections were immersed in 0.3% hydrogen peroxide for 5 min, washed and then incubated for 30 min in affinity-purified human anti-$P2X_7$ antibody[3,12–15] at a concentration of 0.1 μg/ml IgG in Tris-buffered saline (TBS) at pH 7.2. Thereafter, slides were washed in TBS for 5 min, followed by 20 min incubation with 1 : 100 horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (DakoCytomation, Sydney, Australia). All slides were then washed in TBS for 5 min each, dried and mounted in Entellan mounting medium (Merck, Darmstadt, Germany). Approximately

serial sections of each block were stained with a standard haematoxylin and eosin (H–E) protocol. Peptide epitope (10 μM) completely blocked labelling in preadsorption controls while all other normal control sections were negative for $P2X_7$. The non-functional $P2X_7$ receptor antibody is scheduled to become commercially available by the end of 2004 as a monoclonal.

Results

NORMAL PROSTATE

Figure 1A shows a section of tissue stained by H–E from a prostate obtained from a young man established as being genuinely free of any prostate cancer. The diagnosis was made as a result of the continued absence of disease after a 5-year period and of the fact that the tissue was obtained from a man aged 40 presenting with urinary symptoms unrelated to prostate cancer. The serial section stained with anti-$P2X_7$ antibody in Figure 1B shows a total absence of stain indicating no up-regulated cytolytic $P2X_7$ receptor expression and thus no sign of early neoplasia, confirming the original diagnosis that the tissue was normal. Post mortem tissue obtained from young men (age 20–25) who were all free of prostate cancer all showed a similar complete lack of $P2X_7$ receptors in the prostate epithelium ($n = 6$). Similarly, normal breast[3] and skin epithelia are essentially devoid of $P2X_7$ receptors, while melanoma tissue appears heavily stained throughout the lesion.[16] Thus a total of 23 confirmed normal prostate tissues, all devoid of PIN and each sampled over wide regions of the tissue, were found to be devoid of all $P2X_7$ receptors in the acinar epithelial cells. Examples of this total lack of epithelial label for $P2X_7$ receptors are shown in Figure 1C–F.

CONFIRMED PROSTATE CANCER

The contrast with the appearance of the prostate epithelial cells from the 116 confirmed cancer cases was stark. Figure 2A,B shows two serial sections from a typical core through a moderate-grade tumour (Gleason sum score 6) with H–E stain (Figure 2A) and anti-$P2X_7$ antibody stain (Figure 2B). Ubiquitous labelling of $P2X_7$ receptors was found throughout all epithelial cells in the tumour tissue. The direct staining of tumours (Gleason sum scores 4–9) with the antibody to non-functional $P2X_7$ showed that 116/116 of the confirmed tumours exhibited a high density of cytolytic $P2X_7$ receptors in the affected epithelial cells. The receptors were generally condensed onto the apical membrane of the epithelial cells in higher-grade tumours (Gleason sum score 7–9) leaving little intracellular protein. Only when inserted in the cell membrane are $P2X_7$ receptors able to induce apoptosis. No tumour sample of any grade was unlabelled for $P2X_7$. An example is shown in Figure 3. A total of eight cores were collected from a patient identified with a localized moderate-grade tumour sampled in just two cores from the right lobe. Figure 3A,B shows regions of the two cores through tumour tissue in this patient that was labelled for $P2X_7$ receptor. Clear positive cytoplasmic and apical membrane labelling is apparent in the acini within both these cores. The other six cores showed no signs of cancer by H–E, yet each of these six ostensibly unaffected cores (Figure 3C–H) showed a dense concentration of intracellular cytoplasmic $P2X_7$. The protein generally was not expressed on the apical membrane. Each core collected from each of the 116 confirmed cancer cases exhibited distinct epithelial immunoreactivity for $P2X_7$. On average only 30% of the cores collected from each of the 116 patients directly sampled tumour tissue, the remainder sampling morphologically normal prostate tissue.

$P2X_7$ RECEPTOR EXPRESSION IN EARLY CANCER

The changing location and density of $P2X_7$ receptors in prostate epithelium was followed by examining all the biopsies collected prior to the final confirmation of cancer in the 116 confirmed cancer cases. Many of these cases, particularly those collected just 3–6 months before final positive identification of the cancer, were considered false negatives, as the tumour that must have been present at the earlier biopsy was simply missed in the process of sampling the tissue, a common occurrence with small confined tumours. All these cases displayed a characteristic appearance identical to that shown in Figure 3C–H. A distinct universal cytoplasmic intracellular distribution of $P2X_7$ receptors was found in all the epithelial cells even though overall cellular morphology appeared entirely normal by H–E.

An even earlier appearance of $P2X_7$ receptors in prostate epithelial cells was found in tissue biopsied between several months and 6 years prior to the positive confirmation of prostate cancer in the cohort of 116 patients. Some of these patients had exhibited PIN at initial biopsy. These much earlier samples showed apparently normal morphology in all sampled areas, with no sign of tumour in any core. An example is shown in the H–E-stained section in Figure 4A. However, staining for $P2X_7$ receptors in a serial section (Figure 4B) clearly showed that essentially all the

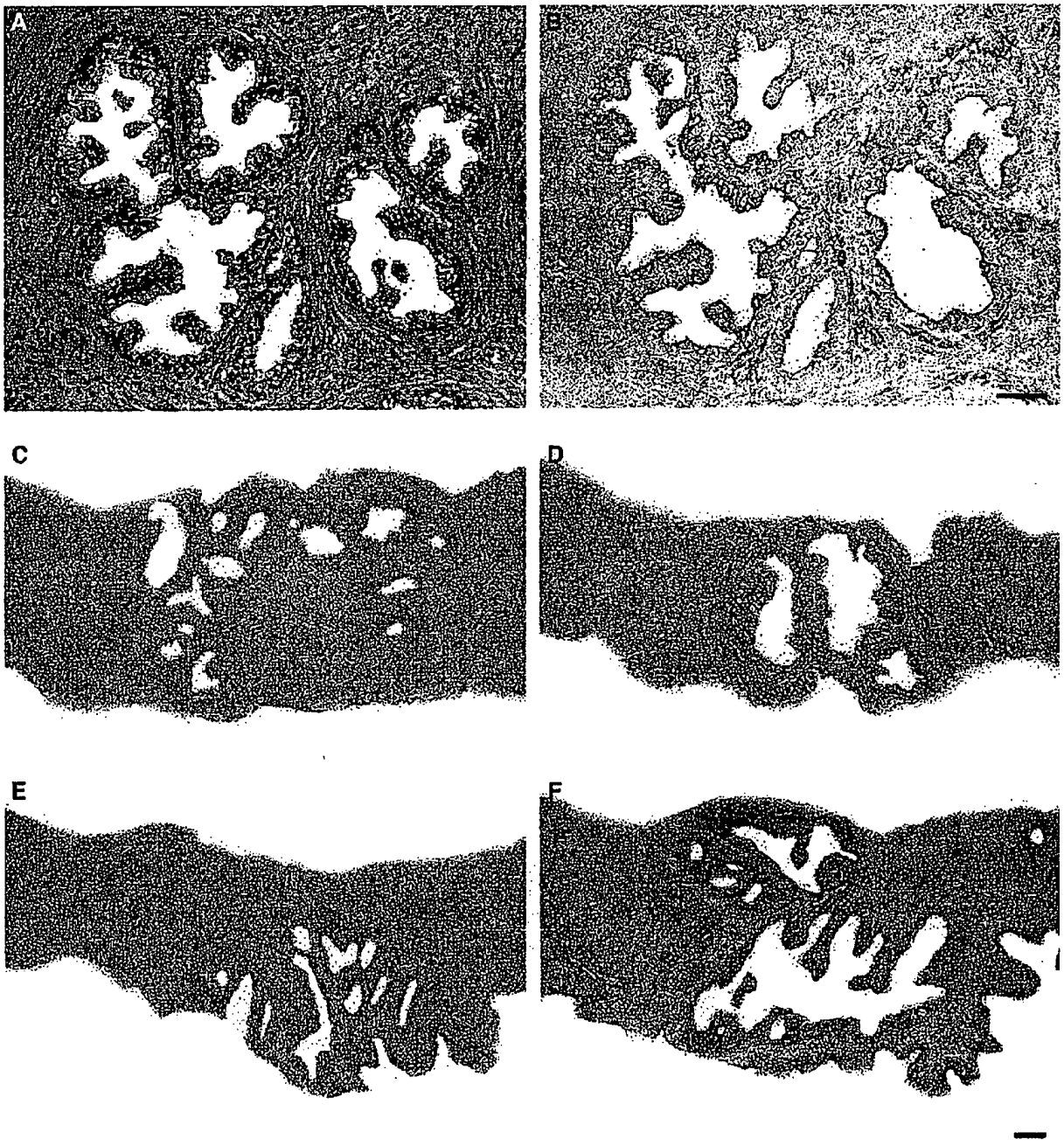


Figure 1. A,B, Serial sections of a prostate core from a patient with no early neoplasia or tumour and who was confirmed to be tumour free for 5 years after this biopsy was collected. A, H–E stain. B, The corresponding serial section is devoid of $P2X_7$ label. Identical results were obtained in normal tissue from young patients obtained at post mortem. C–F, Sections from four different cores collected from a patient subsequently found to be free of prostate cancer. All cores are unlabelled for $P2X_7$. Bars are 50 μm.

epithelial cell nuclei had begun to express $P2X_7$ (see arrows). Figure 4C–F shows areas from four cores obtained from a patient with no sign of cancer at the time of biopsy but who was diagnosed with cancer at a second biopsy investigation 3 years later. Clear nuclear $P2X_7$ label was found in every one of the initial set of six cores taken throughout this prostate. The appearance of $P2X_7$ protein in the epithelial cell nuclei

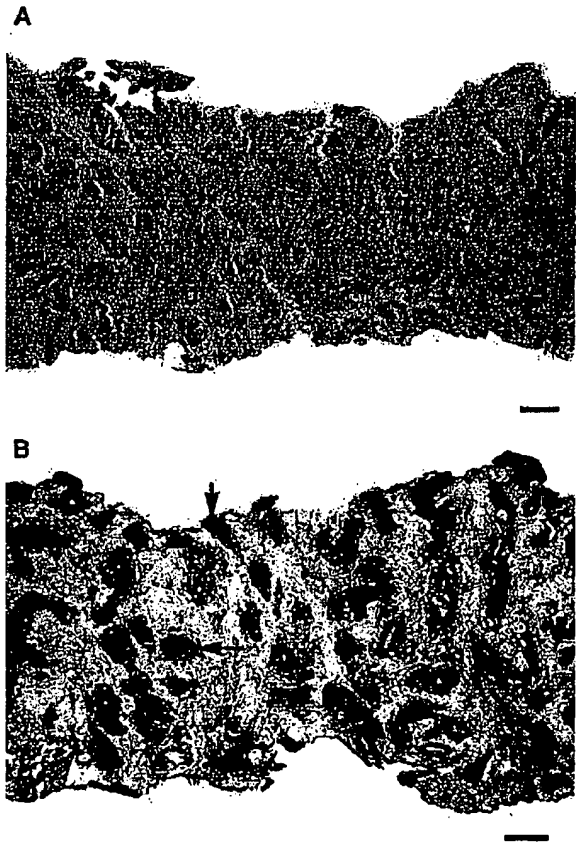


Figure 2. A,B, A core from a patient with moderate cancer (Gleason grade 6). The corresponding $P2X_7$ label shows that all receptors tend to condense on the apical membrane (arrow) of the acinar epithelial cells from within the cytoplasm. Every prostate tumour directly sampled in the core biopsies ($n = 116$) showed an identical pattern of staining in the epithelial cells, comparing starkly with all normal tissue that was all devoid of stain. Bars are 50 μm.

in these prostates appeared to be widespread from a very early stage of developing prostate cancer, before any tumour was detectable by H–E. There were very few acini that did not show $P2X_7$ expression by all luminal epithelial cell nuclei. Intermediate cases were found in which nuclear and cytoplasmic label was coextensive. Of the 116 patients confirmed with cancer, 38 exhibited nuclear receptor expression in tissue collected at earlier biopsies where no trace of cancer was found. They fell into two distinct groups. A total of 17 (44%) were confirmed with cancer by H–E analysis an average of 9 months later (range 6–15 months), while the remaining 21 (56%) were confirmed with cancer an average of 4 years later (range 2–6 years). The transformation rate between nuclear stain and morphologically distinct cancer was an indication of how rapidly the tumour developed. In rapidly developing cancers, the nuclear stain recorded at initial biopsy was replaced with cytoplasmic and apical membrane stain at the later biopsy in as short a time as several months. Several clinical indications such as rapid rise in PSA and/or suspicious digital rectal examination (DRE) alerted the clinicians to the need to quickly re-biopsy in these cases. Many other cases took several years for the prostatic luminal epithelium to undergo the transition in receptor distribution from nucleus to membrane.

This field effect showed that $P2X_7$ protein appeared in the nuclei throughout the prostate epithelium at least several months and up to 6 years before cancer was positively identified by H–E-stained biopsy. Moreover, this same field effect showed that intracellular cytoplasmic $P2X_7$ protein was always present in apparently normal tissue located well away from the site of the confirmed tumour. Tissue sections obtained from radical prostatectomies similarly showed widespread $P2X_7$ cytoplasmic staining throughout the entire sections and not just the sections through the tumour. There were no particular zonal differences found.

Discussion

The current study further validates earlier work using $P2X_1$ and $P2X_2$ to identify patients at risk of developing prostate cancer.[5,6] The study supports the suggestion that major changes in calcium regulatory mechanisms anticipate morphological changes associated with developing moderate- to high-grade neoplasia over times that range from as little as 6 months to 6 years. The faster the tumour grew the faster was the transition time between the appearance of nuclear protein label and the spread of the receptors to the cytoplasm and luminal epithelial cell membrane. $P2X_7$ cytolytic receptors were not found in any normal prostate epithelium in any section taken from 23 normal prostates, whereas the receptors were found in every section examined from 116 confirmed prostate cancer cases (Gleason sum scores 4–9); over 600 biopsied sections in all. In the highest grade prostate cancers (Gleason sum scores 7–9), where sections were taken through the tumour tissue, the pattern of receptor staining showed a distribution largely confined to the apical membranes of the epithelial cells in each acinus, presumably in a failed attempt to induce apoptosis in the affected cancer cells. In more moderate grade cancer tissue (Gleason sum scores 4–6), many of the receptors were found to be intracellular with a dense cytoplasmic distribution in the affected epithelial

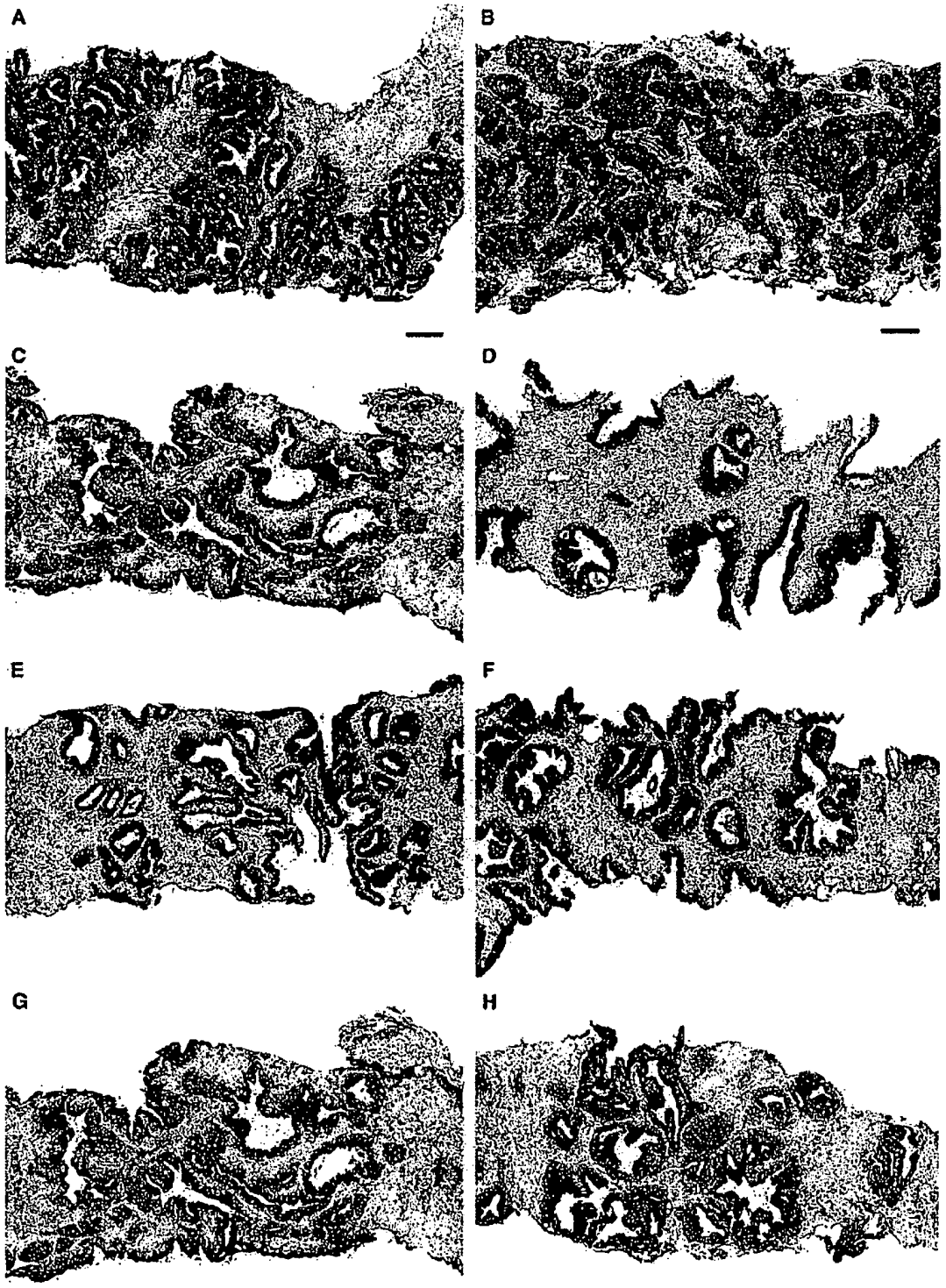


Figure 3. Sections from each of eight cores collected from a patient with prostate cancer and labelled with anti-$P2X_7$ antibody. A,B, Cores taken directly through the tumour. C–H, Sections from six different cores that missed the tumour. Each shows clear cytoplasmic epithelial cell label for $P2X_7$. All epithelial cells are labelled for $P2X_7$. Bars are 50 μm.

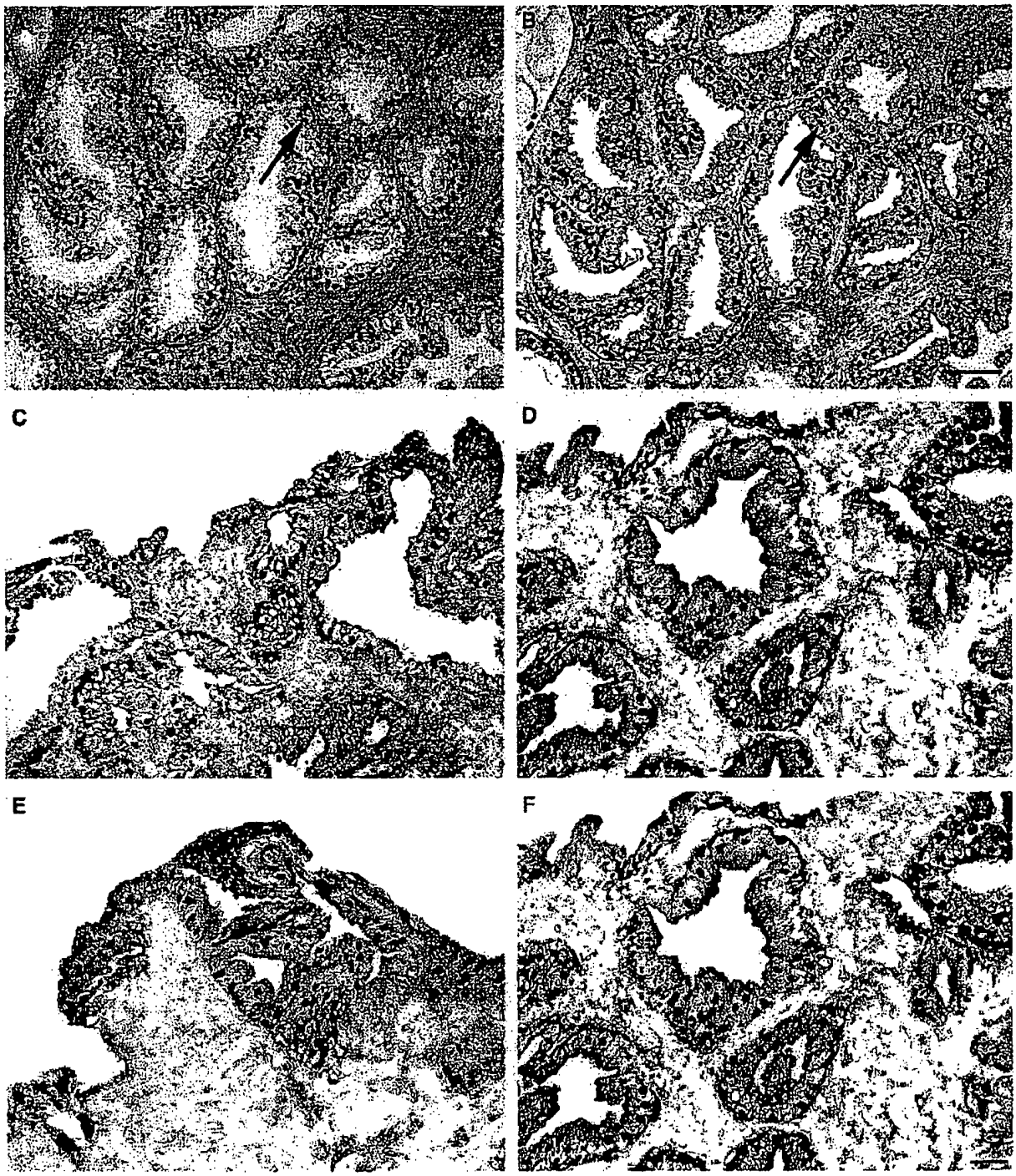


Figure 4. A,B, Serial sections of apparently normal prostate tissue showing a distinct pattern of $P2X_7$ receptor expression in each of the epithelial cell nuclei (arrows). All such cases ($n = 38$) developed clinically significant cancer within 6 years with nearly half displaying obvious tumour within 15 months. Bars are 50 μm. C–F, Sections from each of four cores labelled with anti-$P2X_7$ antibody collected from a patient 3 years before histological identification of a medium-grade contained prostate tumour was made. Distinct nuclear localization of the protein in the epithelial cells in all cores is apparent. Bars are 30 μm.

cells suggesting a high concentration of intracellular receptor not yet deployed on the cell surface. This same high intracellular concentration of receptors was found in apparently morphologically normal tissue well removed from the tumour margin, making detection of the tumour straightforward in those cases where the tumour may not have been sampled directly by the biopsy needle. No case of prostate cancer in this randomly selected cohort of 116 exhibited a different pattern of receptor distribution. Thus, no section of tissue collected from any confirmed tumour case displayed any acini that were not heavily labelled with anti-$P2X_7$ receptor antibody even in areas well removed from the identified tumour, including areas in the opposite lobe. The majority of this patient cohort have received or will soon receive major intervention for their cancer, including radical prostatectomy and radiotherapy. Thus most of the cancers detected at final biopsy were of the category requiring clinical intervention, either immediately or soon thereafter. All such cases requiring clinical intervention displayed receptor expression in epithelial cell cytoplasm and apical membrane. In contrast, very few unimportant or latent cancer conditions were selected in this cohort. Rather, these few cases fell into the category in which the receptor label apparently remained essentially nuclear in character.

This distinctly separate early stage of receptor distribution was seen in 38 of the 116 confirmed cases in the sets of biopsies collected at times that preceded tumour identification by several months to 6 years. No tissue that contained an identified clinically significant tumour exhibited this pattern of epithelial cell nuclear stain. Yet each of the 38 cases identified with $P2X_7$ confined to the epithelial cell nuclei developed into confirmed prostate cancer. The observed transition times between this early manifestation of receptors in the epithelial cell nuclei and detection of tumour fell into two distinct categories: an average of 9 months or an average of 4 years. These distinct categories suggest two broad categories of clinically significant tumour progression. Thus about half the observed tumours from this cohort appeared quite rapidly, while onset of the others was about five-fold slower. Because of the close monitoring of this cohort of patients very few of the tumours were first detected by needle biopsy with a Gleason sum score in excess of G6.

The pattern of receptor expression therefore appears capable of accurately identifying whether prostate cancer is absent (as defined here by an absence of $P2X_7$ receptors in the epithelial cells), is not yet clinically significant, may be classed as latent (as defined here by $P2X_7$ receptors confined within epithelial cell nuclei), or is already present in a clinically significant form (as indicated by the widespread appearance of intracellular $P2X_7$ receptors in the cytoplasm of the epithelial cells lining the acini). The presence of nuclear $P2X_7$ appears to be the initial response of cells to the need to boost the apoptotic mechanisms that are required to counter increased cell proliferation signals. An apoptotic mechanism operating through $P2X_7$ cytolytic receptors cannot occur until the protein is effectively assembled on the cell surface. However, the need for apoptotic pathways to be up-regulated in prostate tissue that detects altered proliferation signals is unsurprising. When this nuclear staining pattern was observed in tissue from a single biopsy it was also found in all other biopsies from the same patient, indicating that the up-regulation of $P2X_7$ in prostate epithelial cell nuclei is both rapid and widespread. Few samples were observed in which the nuclear stain was found alongside unstained acini.

The later stages of tumour development in which $P2X_7$ was found to have migrated into the cell cytoplasm and on to the plasma membrane were observed to occur much more slowly in regions remote from the actual tumour tissue. It was common to find regions in which both nuclear and cytoplasmic immunoreactivity were coextensive in very low-grade cancer cases (Gleason sum score 4), but this pattern was still present in epithelial cells that were remote from some much higher-grade tumours (Gleason sum score 7–8). The presence of every clinically significant tumour in the prostate, regardless of the Gleason grade, tumour size or the age of the patient, was characterized by clear cytoplasmic labelling for $P2X_7$ throughout the entire prostate epithelium ($n = 116$). Every tumour showed distinct cytoplasmic reactivity for $P2X_7$ in all the sampled acini irrespective of whether the acinus was a part of the tumour, was immediately adjacent to the tumour, or was in an otherwise normal region in the opposite lobe to the tumour. This is clearly seen in the example shown in Figure 3, in which a similar staining pattern is observed in all eight biopsy cores even though only two cores were taken through the tumour (Figure 3A,B). This method therefore appears to provide a reliable detection of the presence of clinically significant prostate tumours that are already present but not directly sampled with the biopsy needle, perhaps located in the difficult to reach apical lobe. The presence of the widespread cytoplasmic label for $P2X_7$ in biopsied tissue that appears morphologically normal seems to indicate the need either for an immediate set of more extensive repeat biopsies or else other continued close observation to detect the presence of the tumour that has gone unsampled.

Data obtained by $P2X_7$ immunolabelling appears to be unrelated to that of H–E staining other than in tissue from obvious tumour. Instead, the appearance of the potential cytolytic $P2X_7$ receptors flags underlying metabolic changes that precede the histological development of neoplasia well before the appearance of morphological changes associated with tumour development somewhere in the prostate, perhaps several centimetres from the affected epithelial cells. In contrast, recent work on superficial spreading melanoma ($n = 80$) has shown that anti-$P2X_7$ antibodies stain keratinocytes and melanocytes only in the affected tumour tissue with no field effect such as that evident in the prostate.[16] Like prostate, normal skin did not express the receptors. Thus, both skin and prostate cells up-regulate the expression of the apoptotic receptor in cancer cells without being able successfully to counteract increased cell proliferation.[16] The widespread labelling of telomerase-associated protein (hTP1) in the prostate epithelium in which a tumour was present[6] supports the observation that proliferation factors, perhaps including TP1,[6] act to overwhelm apoptosis. The $P2X_7$ apoptotic receptors become up-regulated in response to systemic and perhaps hormonal signals in the prostate to prepare for the onset of developing cancer. These apoptotic receptor proteins appear to be up-regulated throughout the prostate but are not deployed on the apical membrane of the epithelial cells except in those areas contained within the tumour. There is now clear evidence that these receptors are non-functional[15] in breast[3] as well as in skin[16] and prostate. The receptor deployment on the cell membrane is clearly unsuccessful, with the tumour gradually spreading and the affected epithelial cells unable to undergo apoptosis. This stage-related up-regulation of receptors in advance of the appearance of tumour indicates that the positive staining never indicates a false-positive result, with the process of neoplastic transformation taking several months to many years. No clinically significant tumour was detected by biopsy when the $P2X_7$ receptors were confined to the epithelial cell nuclei ($n = 38$), whereas no tissue in which a clinically significant tumour was found, however, localized, ever lacked cytoplasmic stain throughout both the tumour and adjacent tumour-free regions ($n = 116$).

Tumours that were rapidly developing could be differentiated from those that developed more slowly by observing the transition time between the appearance of $P2X_7$ receptors confined within the epithelial cell nuclei and receptors found throughout the cytoplasm. In this necessarily retrospective study, a rapid transition time (average 9 months) between nuclear and cytoplasmic receptor expression was associated with rapid tumour detection, while slower transition times (average 4 years) were associated with commensurately slower tumour detection times. The presence of a field effect underlying prostate cancer also is suggested by the strong genetic similarities seen between cancer and multifocal precursor lesions such as PIN.[17] These genetic similarities suggest that clonal expansion of precursor lesions such as PIN may account for the multifocal occurrence of prostate tumours.

Detection of the $P2X_7$ receptors does not depend on tumour type, Gleason grade, patient age or the presence or absence of preneoplastic conditions such as PIN. Moreover, patients found to be suffering from benign prostatic hyperplasia who do not show an up-regulation of $P2X_7$ receptors in the prostatic epithelium appear to be entirely free of any prostate cancer. These findings may provide new insights into mechanisms for controlling prostate cancer and may prove beneficial in monitoring those men who present with elevated serum PSA but in whom the biopsied tissue appears normal by H–E. The rate of progress of their disease may be followed by reference to the rate of transformation in $P2X_7$ receptor location. Ideally, this method should also be applied to prospective cohorts of patients followed over several years with the aim of separating patients with latent cancers not requiring clinical intervention (presumably those with steady-state nuclear label) from those cancers with cytoplasmic label that are clinically significant, requiring intervention.

Acknowledgement

This study was supported by the National Health and Medical Research Council of Australia.

References

1. Sakr WA, Grignon DJ, Haas GP *et al.* Age and racial distribution of prostatic intraepithelial neoplasia. *Eur. Urol.* 1996; 30; 138–144.
2. A report of the Australian Cancer Network Working Party on Management of Localised Prostate Cancer (http://www.health.gov.au/nhmrc/publicat/pdf/cp70.pdf), September 2000; 1–121.
3. Slater M, Danieletto S, Gidley-Baird A, Teh LC, Barden JA. Differentiation between cancerous and normal hyperplastic lobules in breast lesions. *Breast Cancer Res. Treat.* 2004; 83; 1–10.
4. Dethlefsen SM, Raab G, Moses MA *et al.* Extracellular calcium influx stimulates metalloproteinase cleavage and secretion of heparin-binding EGF-like growth factor independently of protein kinase C. *J. Cell. Biochem.* 1998; 69; 143–153.
5. Slater M, Delprado WJ, Barden JA. Detection of preneoplasia in histologically normal prostate biopsies. *Prost. Cancer Prostat. Dis.* 2001; 4; 92–96.
6. Slater MD, Lauer C, Gidley-Baird A, Barden JA. Markers for the development of early prostate cancer. *J. Pathol.* 2003; 199; 368–377.

7. Matthews P, Jones C. Clinical implications of telomerase detection. *Histopathology* 2001; 38; 485–498.
8. Lin Y, Uemura H, Fujinami K *et al.* Detection of telomerase activity in prostate needle-biopsy samples. *Prostate* 1998; 36; 121–128.
9. Ueda M, Ouhtit A, Bito T *et al.* Evidence for UV-associated activation of telomerase in human skin. *Cancer Res.* 1997; 57; 370–374.
10. Di Virgilio F, Chiozzi P, Ferrari D *et al.* Nucleotide receptors: an emerging family of regulatory molecules in blood cells. *Blood* 2001; 97; 587–600.
11. Wiley JS, Gu BJ, Zhang W *et al.* Genetic polymorphisms of the human $P2X_7$ receptor and relationship to function. *Drug Dev. Res.* 2001; 53; 72–76.
12. Gu BJ, Zhang W, Worthington RA *et al.* A Glu-496 to Ala polymorphism leads to loss of function of the human $P2X_7$ receptor. *J. Biol. Chem.* 2001; 276; 11135–11142.
13. Kukley M, Barden JA, Steinhäuser C *et al.* Distribution of P2X receptors on astrocytes in juvenile rat hippocampus. *Glia* 2001; 36; 11–21.
14. Moore KH, Ray FR, Barden JA. Loss of expression of purinergic $P2X_3$ and $P2X_5$ receptors in adults with overactive detrusor contractility and urge incontinence. *J. Neurosci.* 2001; 21; U17–U22.
15. Barden JA, Sluyter R, Gu BJ *et al.* Specific detection of non-functional $P2X_7$ receptors in HEK293 cells and B-lymphocytes. *FEBS Lett.* 2003; 538; 159–162.
16. Slater M, Scolyer RA, Gidley-Baird A, Barden JA. Increased expression of apoptotic markers in melanoma. *Melanoma Res.* 2003; 13; 137–147.
17. Foster CS, Bostwick DG, Bonkhoff H *et al.* Cellular and molecular pathology of prostate cancer precursors. *Scand. J. Urol. Nephrol. Suppl.* 2000; 19–43.

Exhibit C

Breast Cancer Research and Treatment 83: 1–10, 2004.

Report

Differentiation between cancerous and normal hyperplastic lobules in breast lesions

Michael Slater[1], Suzanne Danieletto[2], Margaret Pooley[3], Liew Cheng Teh[1], Angus Gidley-Baird[1], and Julian A. Barden[1]

[1]*Department of Anatomy and Histology, Institute for Biomedical Research, The University of Sydney, Sydney;* [2]*Department of Pathology, Sydney Adventist Hospital;* [3]*Specialist Medical Centre, Wahroonga, Australia*

Key words: apoptosis, breast cancer, early diagnosis, $P2X_7$ receptors

Summary

Determining the risk that a particular area of hyperplastic breast tissue will progress to cancer is difficult and is currently expressed only as a general risk factor within the population. Using an antibody against the apoptotic purinergic receptor $P2X_7$, we examined 40 cases each of the following histological categories: normal, moderate, florid and atypical hyperplasia, lobular carcinoma *in situ*, ductal carcinoma *in situ*, invasive lobular and invasive ductal carcinoma. These were previously diagnosed by H&E and supplied by clinical laboratories as tissue sections. Normal and mildly hyperplastic epithelium was devoid of the cytolytic $P2X_7$ receptors whereas all epithelial cells in all cases of *in situ* or invasive lobular or ductal carcinoma labelled intensely. The lobular and ductal *in situ* cases labelled intracellularly while the invasive epithelial cancer cells showed intense cell surface label indicating an attempt was being made to induce apoptosis. All these receptors however are non-functional and thus unable to induce apoptosis. Approximately 10% of all hyperplastic lobules examined in the biopsied tissue, regardless of H&E classification, labelled for $P2X_7$, which is suggestive of early metabolic cancerous change. The acini within lobules were either completely labelled with $P2X_7$ or were completely devoid of the receptor. A potential advantage of this method lies in identifying early cancerous change in hyperplastic lobules and in establishing the true extent of cancerous spread in infiltrating lesions, thus facilitating the task of reporting clear surgical margins.

Introduction

Histological and cytological features as demonstrated by haematoxylin and eosin (H&E) staining of tissue sections currently form the basis for the classification of breast cancer. Normal acini are composed of a layer of myoepithelial cells adjacent to the basement membrane that interact with the underlying stroma and an epithelial layer with secretory and absorptive functions on the ductal aspect [1].

Epithelial hyperplasia, sometimes called papillomatosis or epitheliosis, is a relatively common benign microscopic lesion in the breast in women over 30 years of age. The condition involves an increase in the number of layers of epithelial cells through increased proliferation or reduced apoptosis. These cells are heterogeneous and proliferate in layers that extend to ultimately fill the duct lumen. Mild epithelial hyperplasia is defined as consisting of fewer than four cell layers. The terms moderate to usual epithelial hyperplasia describe an increasing number of layers. Atypical lobular hyperplasia refers to hyperplastic cells that resemble lobular carcinoma *in situ* (LCIS) in acinal organisation. Hyperplasia ultimately takes the form of a solid mass of heterogeneous cells that fill and distort the acinus and duct lumen.

It is not possible to determine if lobules containing moderate, usual or atypical hyperplasia will progress to cancer based solely on H&E stained slides. Several studies have shown an unacceptably high level of observer variability in estimating the type and degree of epithelial hyperplasia [2]. Possibly as a result of

this uncertainty, only estimations of general risk to the population are used. Moderate or florid hyperplasia without atypia is considered to carry a slight (1.5–2.0-fold) increase in risk of later developing cancer, while in atypical hyperplasia the risk is given as 4–5-fold that of the general population. Ductal carcinoma *in situ* (DCIS) and LCIS carry an 11-fold increased risk for breast carcinoma [3, 4]. It would be clearly advantageous to be able to differentiate between cells forming low-risk tumours and those that demonstrate the potential for becoming invasive.

The neoplastic transformation of normal breast cells is thought to result in part from a loss of the normal regulation of cell numbers, resulting in hyperplasia. Breast cancer is thought to develop from non-invasive precursor lesions, although the earliest steps of neoplastic transformation are still undefined. Genetic instability in clonal populations of cells is common in atypical hyperplasia. Progression to cancer often includes the increased expression of oncogenes, decreased expression of tumour-suppressor genes, loss of cell adhesion and disruption to cellular biochemistry. Alterations in cellular morphology occur later as a result of these initial changes. In established cancer, changes include the expression of angiogenic factors and proteases that facilitate tissue invasion. These malignant phenotypes are thought to be due to an accumulation of the above changes, rather than an orderly progression between stages [5–7].

As a result, most human breast cancers appear to develop over long periods from pre-existing benign lesions although the early biochemical changes involved in this evolution are not fully understood [8]. Morphological analyses of H&E-stained breast cancer cells suggest that this is a multistep process. Apparently benign lesions progress to various categories of hyperplasia, which represent the initial stages of uncontrolled and possible neoplastic growth. The next stages are carcinoma *in situ*, invasive carcinoma, and ultimately metastasis. Such processes have been documented in many other malignancies. This view suggests that usual ductal hyperplasia (UDH) may be a precursor of atypical ductal hyperplasia (ADH). Conversely, another prevalent view is that UDH is not part of the neoplastic progression but represents a benign proliferation of ductal epithelial cells. ADH does however represent the first step in a clonal neoplastic expansion [9].

The prognostic significance of histological change as demonstrated by H&E stain has been extensively studied and forms the basis of current diagnostic criteria. More recently, other markers of early cancerous change (i.e., unrelated to the expression of the cytolytic $P2X_7$ receptors described in this study) have been discovered. A loss of the genes 16q and 17p may also play an early role in breast carcinogenesis [10]. Altered protein expression from several proliferation and apoptosis-related genes are a known factor in the development of breast cancer and are found in UDH [11]. The biggest change in expression of proliferation and apoptosis related proteins appear to occur during the transition from UDH to DCIS [12]. This implies that 'benign' hyperplasia may indeed be a first event in breast oncogenesis. In a study of 66 human breast carcinomas and adjacent peritumoural tissues, tumour markers human erythrocyte glucose transporter 1 (GLUT1) and fatty acid synthase (FAS) were observed in non-neoplastic tissues that were adjacent to mammary carcinomas [13]. Hypermethylation of the 14-3-3-sigma gene occurs at an early stage in the progression of breast cancer, and has been noted in apparently normal epithelium adjacent to breast cancer. A loss of expression of 14-3-3 sigma is therefore possibly another early event in neoplastic transformation [14].

The clonal nature of neoplastic lesions such as *in situ* and invasive breast cancer has been widely proven by several proliferative, genetic or other malignancy-associated markers. The breast is organised into distinct stem cell-derived monoclonal patches and the normal terminal ductal lobular units (TDLU) are monoclonal in origin. Any proliferative lesion arising within such a pre-existing clonal patch should therefore be clonal, irrespective of whether it originates from one or more patch cells [15]. Loss of heterozygosity (LOH), a genetic change frequently detected in cancer, has been noted in 'benign' epithelial foci in the breast. LOH also occurs frequently in fibrocystic change, which suggests that foci of apocrine metaplasia can share a genetically altered precursor cell with an associated carcinoma [16].

In previous studies on the early neoplastic biochemical changes in another epithelial cell (prostate) cancer [17], we established that the expression of several purinergic receptors precedes any visible markers of cancer as visualised by H&E stain by up to 6 years. More recently, we discovered that the apoptotic receptor $P2X_7$ is expressed in all melanoma cases investigated, while remaining absent in normal skin epithelium [18]. This receptor has since been confirmed as being non-functional, entirely unable to induce apoptosis in the cells expressing surface

receptor/channels [19]. In the current study, we labelled previously diagnosed (by H&E stain) breast biopsies identified as normal, hyperplastic (mild, florid and atypical), *in situ* and invasive carcinoma with an antibody to the apoptotic calcium channel receptor $P2X_7$ to identify the early biochemical changes of breast cancer. These $P2X_7$ receptors expressed on breast cells have similarly been found to be non-functional.

Materials and methods

Each of the following histological categories ($n = 40$) was investigated: normal, usual, and atypical hyperplasia, LCIS, DCIS, invasive lobular and invasive ductal carcinoma. Each case was previously diagnosed and supplied as tissue sections by clinical laboratories on a random basis accompanied by full case notes and an H&E-stained slide for correlative microscopy. One of the difficulties in this study was that, although each case had an overall diagnosis, the tissue within each block (up to 30 blocks per case) varied markedly. Examples of a variety of histological classifications were present not only in each block, but between blocks from the same case. Consequently, the classification state of each lobule had to be verified by a histopathologist before being included in the study. Despite this precaution, expert opinions varied as to the classification of some hyperplastic lobules.

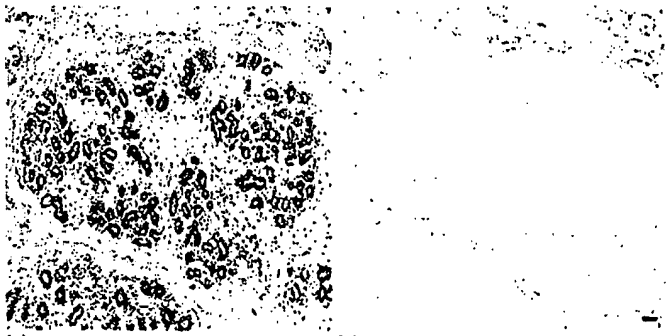
As this was an initial study to establish the $P2X_7$ labelling characteristics compared with the established H&E diagnostic criteria, it was not appropriate to use a blind study. Each case was selected randomly from a list of available patients within each of the listed categories and diagnosed by a qualified pathologist to establish the cancer stage present, using current diagnostic H&E-stain criteria. The affinity-purified $P2X_7$ antibody was prepared as previously described [20]. Each tissue section was immunolabelled as previously described [21]. Identical labelling was observed using the antibody to non-functional receptors [19] verifying the suspicion that the $P2X_7$ apoptotic pathway is blocked in breast cancer. Each slide was de-waxed in two changes of fresh Histoclear for 10 min each and then rehydrated. All sections were immersed in 1% hydrogen peroxide for 5 min, washed and then incubated in anti-$P2X_7$ primary antibody at a concentration of 0.25 μg/mL IgG in PBS for 30 min. Thereafter, slides were washed three times in PBS for 5 min each, followed by a total 30 min incubation with anti-rabbit secondary (Dako). All slides were then washed in PBS for 5 min and visualised using a 0.05% solution of diamino benzidine (DAB) for 5 min, washed, dried and mounted in Entellan mounting medium (Merck). Approximately serial sections were stained with a standard H&E protocol. Peptide epitopes completely blocked labelling in pre-absorption controls. For practical, ethical and legal reasons, only formalin-fixed, paraffin-embedded and previously diagnosed biopsies were available for this study.

Results

Figure 1(a) is a micrograph of a normal TDLU stained by H&E. The acini are composed of two cellular layers, an epithelial layer on the lumenal aspect that has secretory and absorptive functions, and a myoepithelial layer adjacent to the acinal basement membrane. The ducts draining the acini are lined with stratified epithelium. The interlobular stroma is stained magenta by the eosin component of the stain. In serial sections of lobules with this H&E appearance (Figure 1(a)), no labelling for the cytolytic receptor $P2X_7$ was seen (Figure 1(b)).

Approximately 10% of all hyperplastic lobules labelled strongly for $P2X_7$ regardless of the H&E classification, in all biopsied tissue. This label was 'all or nothing' in every acinus within a given lobule, suggesting that the phenomenon spread to neighbouring cells within the individual duct. This finding has also been noted in the prostate [21]. In usual hyperplasia, the proliferating epithelium takes the form of solid masses of cells that distort the acinal structure and obscure the duct lumen (Figures 2 and 3). Within this classification, 88% of lobules ($n = 3987$) displayed no neoplastic-associated biochemical changes as determined by cytolytic $P2X_7$ receptor labelling (Figures 2(a and b)) while the other 12% demonstrated a positive $P2X_7$ receptor label throughout all epithelial cells in the lobule (Figures 3(a and b)). It should be noted however that the receptor expression was intracellular. The receptors had not been transported to the cell membrane where they could be assembled to act as functional apoptotic pores following agonist activation [19].

Tissue sections often contained different types of lobule as classified by H&E. Often, however the tissue sections would contain lobules that were indistinguishable from one another by H&E stain but were either strongly positive or transparent (negative)

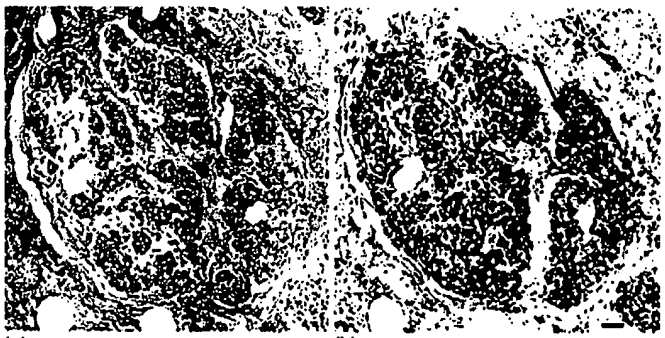


(a) (b)

Figure 1. (a) A micrograph of a terminal duct lobular unit stained by H&E. (b) A serial section of the same lobule as (a) labelled for the cytolytic receptor anti-$P2X_7$. There is no positive label for this receptor in normal tissue. Bar = 100 μm.

(a) (b)

Figure 2. (a) An H&E stained section of a hyperplastic lobule. The proliferating epithelium has taken the form of solid masses of cells that obscure the duct lumen. (b) Serial section of (a) that has been labelled for $P2X_7$ showing no label (i.e., normal) in the cytoplasm and apical epithelium (arrow). Bar = 100 μm.



(a) (b)

Figure 3. (a) An H&E stained section of hyperplastic lobules. The proliferating epithelium has taken the form of solid masses of cells that obscure the duct lumen. (b) A serial section of Figure 2(a) that has been labelled for $P2X_7$ showing an intense label in the cytoplasm and beneath the apical epithelium (arrow). Bar = 100 μm.

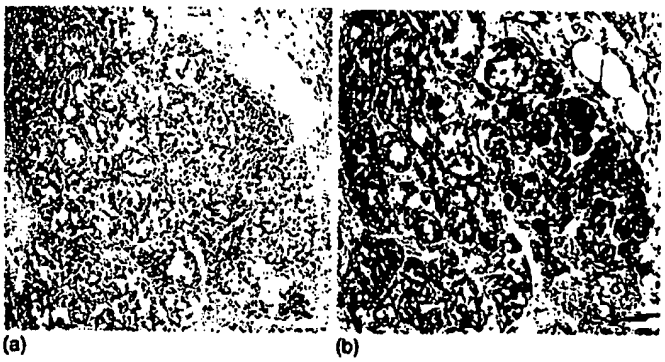


Figure 4. (a) An H&E stained section that shows a lobule diagnosed as atypical hyperplasia. (b) A serial section that shows intense intracellular $P2X_7$ receptor label in this lobule, suggesting the presence of early neoplastic change. Bar = 50 μm.

Figure 5. (a) A low power H&E stained section of an area of tissue diagnosed as mixed normal and atypical hyperplasia. (b) An approximate serial section of the same area labelled for $P2X_7$. Using this marker, some lobules are normal (NL-arrows) and some show positive neoplastic changes in the form of intracellular $P2X_7$ receptors (AR-arrow). Note that this observation is an 'all or nothing' phenomenon at the lobular level. Individual acini conform to the labelling state of their lobule. Bar = 500 μm.

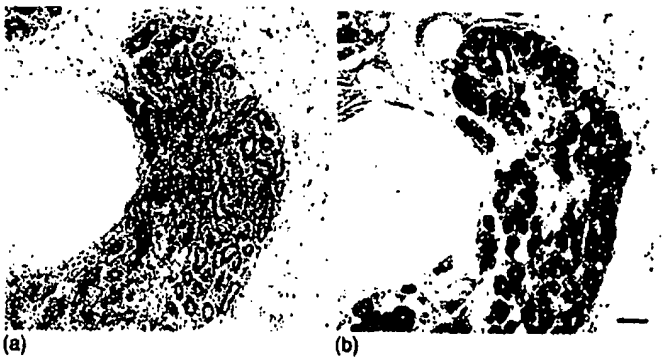


Figure 6. (a) A low power H&E stained section of the lobule labelled 'AR-arrow' in Figure 4(b). While some acini appear normal, others do not. (b) A serial section that shows an intense intracellular $P2X_7$ receptor label in the lobule shown in Figure 4(c). Each acinus within the lobule, regardless of H&E morphology, is labelled for the $P2X_7$ receptor. Bar = 20 μm.

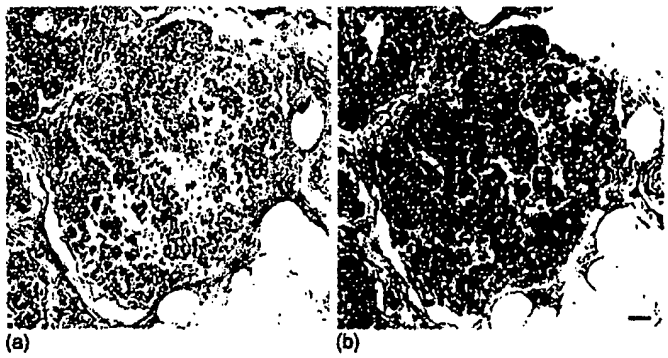


Figure 7. (a) An H&E-stained section previously diagnosed as LCIS. (b) A serial section labelled for the $P2X_7$ cytolytic receptor. All LCIS lobules had an intense intracellular $P2X_7$ label. Bar = 50 μm.

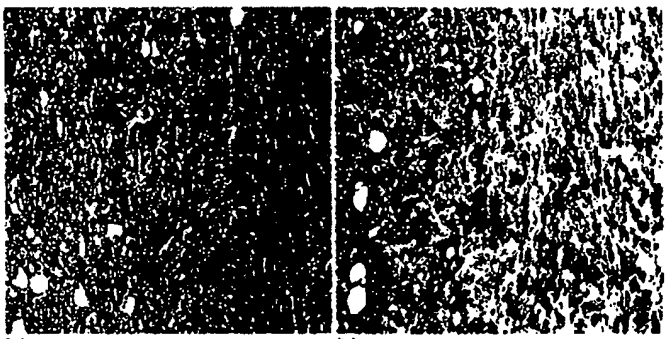


Figure 8. (a) An H&E-stained tissue section previously diagnosed as invasive carcinoma. (b) An approximate serial section of Figure 6(a). Each cell shows an intense cell surface label for $P2X_7$ despite histological degradation. Bar = 50 μm.

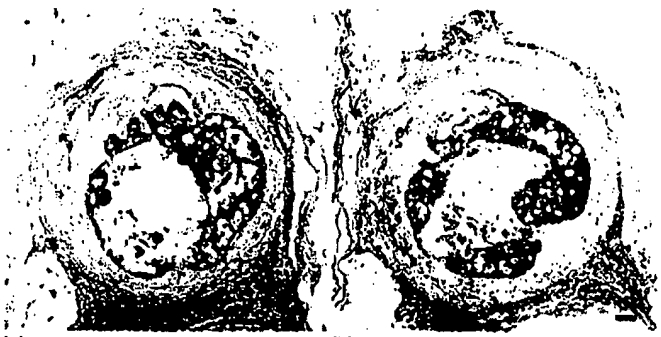


Figure 9. (a) An H&E-stained section of a DCIS of the comedo type. (b) A serial section of Figure 7(a). Despite some histological degradation, an intense intracellular label for $P2X_7$ is visible in all cells. Bar = 100 μm.

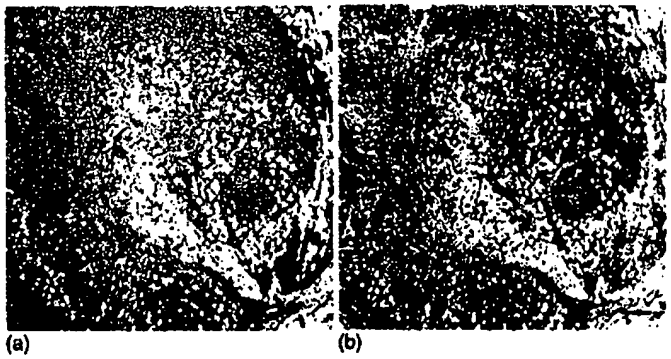


Figure 10. (a) An H&E-stained section of invasive ductal carcinoma. (b) Each epithelial cell surface is intensely labelled for $P2X_7$. Bar = 30 μm.

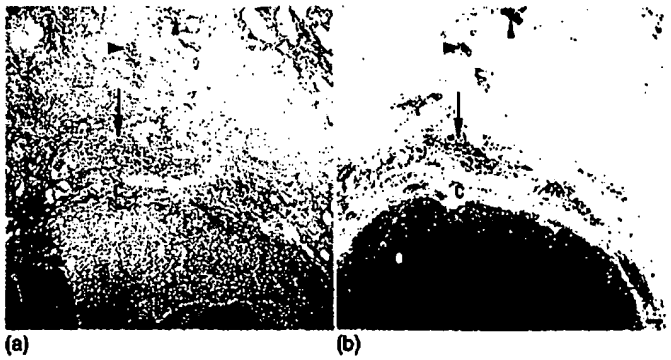


Figure 11. (a) An H&E stained-section of a large area of carcinoma *in situ*. A capsule (C) surrounds the cancerous area. (b) Areas of intracellular $P2X_7$ labelled cells (Figure 9(a and b)-arrows) can be seen at least 1.2 cm from the primary tumour on the upper margin, while all the other margins were found to be entirely clear of label. Cells within the tumour show mostly cell surface label. Bar = 500 μm.

when labelled for the $P2X_7$ receptor. Figure 4(a) shows a lobule clinically diagnosed with atypical hyperplasia. In atypical hyperplasia a total of 90.4% of all lobules were unlabelled, suggesting that they had not been subject to early neoplastic biochemical change ($n = 4328$). However, the remaining 9.6% were strongly positive, indicating possible early potential neoplastic change. Once again, the receptors were found to be intracellular. As transport to the cell membrane had not yet occurred there was not yet even the potential for apoptosis to be induced through stimulation of these receptors. Figure 4(b) demonstrates an intense $P2X_7$ receptor label in a serial section. Both nuclei and cytoplasm in all epithelial cells are labelled.

Figure 5(a) is a low power H&E stained section of an area of tissue diagnosed as comprising mixed normal and atypical hyperplastic lobules. Figure 5(b) is an approximate serial section of the same area labelled for $P2X_7$ showing some lobules are normal (NL-arrows) while some are positively labelled (AR-arrow). The 'all or nothing' nature of this phenomenon is clear in this micrograph. Figures 6(a and b) are high power micrographs of the lobule labelled 'AR' in Figure 5(b). All receptors are found to be intracellular in these tissues.

In tissue that was diagnosed as clearly cancerous by H&E stain, all epithelial cells labelled strongly for $P2X_7$. Figure 7(a) is an H&E-stained section previously diagnosed as LCIS while Figure 7(b) is a serial section labelled for $P2X_7$. All LCIS lobules were strongly labelled with the $P2X_7$ antibodies (Figure 5(b)). While the number of receptors appeared

to be substantially increased in these tissues over the levels observed in moderate and atypical hyperplasia, they remained intracellular.

Figure 8(a) is an H&E-stained tissue section previously diagnosed as invasive lobular carcinoma. All the epithelial cells demonstrate an intense $P2X_7$ label (Figure 8(b)). However, the receptors are mostly deployed on the cell surface where they have the potential to induce apoptosis. They are unable to do so as the pores remain closed as revealed by the binding of the antibody to the non-functional receptors.

Figure 9(a) is an H&E-stained section of a DCIS of the comedo type. Once again an intense label for $P2X_7$ is visible (Figure 9(b)) in all epithelial cells. These cells also show the receptors are almost entirely intracellular.

Figure 10(a) is an H&E-stained section diagnosed as invasive ductal carcinoma. Figure 10(b) shows that all the epithelial cells in the field are intensely labelled for $P2X_7$ as expected. All cells identified as cancerous by H&E were labelled with the $P2X_7$ antibody. Essentially all the receptors in these cells also have been deployed on the cell membrane in an apparently unsuccessful attempt to induce apoptosis.

This simple immunohistochemistry technique also proves useful for determining the extent of tissue invasion from a primary cancer. Figure 11(a) is an H&E-stained section of invasive ductal carcinoma. Areas of $P2X_7$ labelled cells (Figure 11(b)-arrows) can be seen at least 1.2 cm from the primary tumour on the upper margin, while all the other margins were found to be clear of label. These labelled acini otherwise appear unremarkable by H&E. Most of the receptors in these otherwise unremarkable cells are found to be intracellular so that apoptosis cannot yet be induced by this pathway.

Discussion

In this study, $P2X_7$ receptors were not found in either normal or in approximately 90% of hyperplastic lobules. In all forms of lobular, ductal, *in situ* and invasive carcinomas however, labelling was intense but only invasive carcinoma cells displayed surface receptor. These findings were completely independent of hormonal cyclic changes and are consistent with recent studies that show that apoptosis is increased with increased proliferation and is associated with breast cancer [22]. However, the fact that the $P2X_7$ receptors expressed on the cell surface in invasive cancer were non-functional indicates that this potential apoptotic pathway is not available in breast cancer. This simple immunohistochemistry method has more value in identifying early cancerous change in hyperplastic lobules rather than established cancer, as the latter is clearly identifiable by both H&E and cell surface $P2X_7$ staining. Furthermore, cell surface expression of the receptors can be used to verify the invasive state of affected cells.

Mild hyperplasia displayed no $P2X_7$ label in the affected epithelial cells. Usual and atypical hyperplasias had distinct $P2X_7$ receptor labelling patterns that could clearly differentiate between normal and potentially cancerous lobules. These differences were not visible by H&E stain. In usual hyperplastic epithelium, 88% of lobules ($n = 3984$) showed no label while 12% were strongly positive. These receptors remained intracellular. Although they were upregulated, they were not yet transported to the cell surface where they have the potential to induce apoptosis. This observation was essentially an 'all-or nothing' lobular phenomenon. Each cell of an affected acinus was labelled whereas in adjacent normal lobules, no acinal cells were labelled. This result suggests that a fundamental neoplastic change had occurred in 12% of the lobules and that the expression of $P2X_7$ receptors was possibly a protective response to that change, with an attempt to up-regulate apoptosis. The affected lobules may also be connected in the same affected duct.

Atypical lobular and ductal hyperplasia is thought to have a malignancy progression risk that is 4–5 times greater than that of the general population [3, 4, 23]. In the current study, 90.4% of all lobules in tissue clinically diagnosed as atypical hyperplasia did not label for $P2X_7$ cytolytic receptor, suggesting that they had not been subject to the early biochemical changes associated with neoplasia. However, the remaining 9.6% of the total lobules (Figure 5(b) AR-arrow) were strongly and uniformly labelled with intracellular receptor, possibly indicating the presence of early *in situ* cancer somewhere within this ductal unit. Moreover, the connectivity between the labelling of a minority of lobules in UDH and the likely progression of these affected lobules to ADH and then DCIS appears to be well established from the results shown here by means of the upregulation of the intracellular $P2X_7$ receptors.

All cases of diagnosed *in situ* and invasive cancer exhibited an intense nuclear and cytoplasmic $P2X_7$ label in all epithelial cells with the invasive cancers progressing to the stage of deploying the receptors on the cell surface. Figure 11, from a case of invasive

ductal carcinoma, demonstrates that receptor labelling can be used to determine the extent of neoplastic invasion. Figure 11(b) shows that pre-cancerous acini are present at least 1.2 cm from the primary tumour on this margin with all other margins having been shown to be clear of label, suggesting that not all affected tissue was excised.

DCIS represents approximately half of all breast carcinomas detected by mammographic screening. The lesion is divided into several architectural subtypes as defined by H&E stain. As a whole though, DCIS consists of a malignant population of cells that lack the capacity to invade through the basement membrane. They may however spread throughout the ductal system thereby involving an entire sector of the breast. DCIS has several morphological variants [24]. LCIS involves proliferation of a monomorphic population of cells in the terminal ducts or acini. The cells are larger than normal and often signet ring cells containing mucin are present. Compared with ductal carcinoma, lobular carcinoma is found less frequently, representing only 1–6% of all breast carcinomas. Invasive carcinoma develops from LCIS in only 25–35% of cases [25]. In the current study, all cases of DCIS and LCIS as well as invasive carcinoma, showed an intense up-regulation of $P2X_7$ receptors in all affected epithelial cells, but significantly the more dangerous invasive cells could be differentiated from the *in situ* tumours by means of the surface expression of the receptors observed only in the invasive cells.

We have shown that labelling for $P2X_7$ can distinguish between cells that have been transformed biochemically from those that are biochemically normal, an observation not possible using H&E staining. Precise identification of early biochemical changes associated with signs of preneoplasia in usual and atypical hyperplastic cells is therefore clear and unambiguous. All normal cells are devoid of the receptor while all cancer cells and those cells that are turning cancerous are uniformly and heavily labelled for the apoptotic receptor. Only the invasive cells show surface deployment of the receptors. Thus there is the potential to discriminate between cells forming low-risk tumours and cells that have more invasive potential. As we have previously shown in the prostate, this method allows early and appropriate intervention where the H&E findings might be ambiguous. Conversely, anxious patients with non-cancerous lesions or those with no sign of invasive cancer can be confidently reassured. This method also identifies the true extent of precancerous spread in infiltrating lesions, to facilitate the accurate reporting of clear surgical margins. A multiple-time point prospective study is necessary to establish these findings as an independent predictor of risk.

Acknowledgement

Dr Michael Slater is supported by a National Health and Medical Research Council of Australia Industry Research Fellowship.

References

1. Jarasch ED, Nagle RB, Kaufmann M, Maurer C, Bocker WJ: Differential diagnosis of benign epithelial proliferations and carcinomas of the breast using antibodies to cytokeratins. Hum Pathol 19: 276–289, 1988
2. Bodian CA, Perzin KH, Lattes R, Hoffmann P: Reproducibility and validity of pathologic classifications of benign breast disease and implications for clinical applications. Cancer 71: 3908–3913, 1993
3. Batori M, Gallinaro LS, D'Urso A, Ruggeri M, Lorusso R, Forte A, Fierro N: Papillomatosis and breast cancer: a case report and a review of the literature. Eur Rev Med Pharm Sci 4: 99–103, 2000
4. Jacobs TW, Connolly JL, Schnitt SJ: Nonmalignant lesions in breast core needle biopsies: to excise or not to excise? Am J Surg Pathol 26: 1095–1110, 2002
5. Fearon ER: Human cancer syndromes: clues to the origin and nature of cancer. Science 278: 1043–1050, 1997
6. Cohen SM, Ellwein LB: Genetic errors, cell proliferation, and carcinogenesis. Cancer Res 51: 6493–6505, 1991
7. Connolly J: Principles of cancer pathology. In: Holland J (ed) Cancer Medicine. Williams and Williams, Baltimore, 1997, p 533
8. Allred DC, Mohsin SK, Fuqua SA: Histological and biological evolution of human premalignant breast disease. Endocr-Relat-Cancer 8: 47–61, 2001
9. Russo J, Russo IH: The pathway of neoplastic transformation of human breast epithelial cells. Radiat Res 155: 151–154, 2001
10. Gong G, DeVries S, Chew KL, Cha I, Ljung BM, Waldman FM: Genetic changes in paired atypical and usual ductal hyperplasia of the breast by comparative genomic hybridization. Clin Can Res 7: 2410–2414, 2001
11. Mommers EC, van Diest PJ, Leonhart AM, Meijer CJ, Baak JP: Expression of proliferation and apoptosis-related proteins in usual ductal hyperplasia of the breast. Hum Pathol 29: 1539–1545, 1998
12. Mommers EC, Leonhart AM, Falix F, Michalides R, Meijer CJ, Baak JP, Diest PJ: Similarity in expression of cell cycle proteins between *in situ* and invasive ductal breast lesions of same differentiation grade. J Pathol 194: 327–333, 2001
13. Alo PL, Visca P, Botti C, Galati GM, Sebastiani V, Andreano T, Di Tondo U, Pizer ES: Immunohistochemical expression of human erythrocyte glucose transporter and fatty acid synthase in infiltrating breast carcinomas and adjacent typical/atypical hyperplastic or normal breast tissue. Am J Clin Pathol 116: 129–134, 2001

14. Umbricht CB, Evron E, Gabrielson E, Ferguson A, Marks J, Sukumar S: Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer. Oncogene 20: 3348–3353, 2001
15. Diallo R, Schaefer KL, Poremba C, Shivazi N, Willmann V, Buerger H, Dockhorn-Dworniczak B, Boecker W: Monoclonality in normal epithelium and in hyperplastic and neoplastic lesions of the breast. J Pathol 193: 27–32, 2001
16. Washington C, Dalbegue F, Abreo F, Taubenberger JK, Lichy JH: Loss of heterozygosity in fibrocystic change of the breast: genetic relationship between benign proliferative lesions and associated carcinomas. Am J Pathol 157: 323–329, 2000
17. Slater M, Lauer C, Gidley-Baird A, Barden JA: Markers for the development of early prostate cancer. J Pathol 199: 368–377, 2002
18. Slater M, Scolyer RA, Gidley-Baird A, Barden JA: (2003) Increased expression of apoptotic markers in melanoma. Melanoma Res 13: 1–9, 2003
19. Barden JA, Sluyter R, Gu BJ, Wiley JS: Specific detection of non-functional $P2X_7$ receptors in HEK293 cells and B-lymphocytes. FEBS Lett 538: 159–162, 2003
20. Moore K, Ray F, Barden J: Loss of expression of purinergic $P2X_3$ and $P2X_5$ receptors in adults with overactive detrusor contractility and urge incontinence. J Neurosci 21: U17–U22, 2001
21. Slater M, Delprado WJ, Murphy CR, Barden JA: Detection of preneoplasia in histologically normal prostate biopsies. Prost Cancer Prost Dis 4: 92–96, 2002
22. Parton M, Dowsett M, Smith I: Studies of apoptosis in breast cancer. Br Med J 322: 1528–1532, 2001
23. Page DL: Cancer risk assessment in benign breast biopsies. Hum Pathol 17: 871–874, 1986
24. Lennington WJ, Jensen RA, Dalton LW, Page DL: Ductal carcinoma *in situ* of the breast. Heterogeneity of individual lesions. Cancer 73: 118–124, 1994
25. Rosen PP, Oberman HA: Tumors of the Mammary Gland. Armed Forces Institute of Pathology, Washington DC, 1993

Address for offprints and correspondence: Dr Julian A. Barden, Department of Anatomy and Histology, Anderson Stuart Building F13, Institute for Biomedical Research, The University of Sydney, Sydney NSW 2006, Australia; *Fax:* +61-2-9351-6878; *E-mail:* julian@anatomy.usyd.edu.au

Exhibit D

Increased expression of apoptotic markers in melanoma

Michael Slater[a], Richard A Scolyer[b,c,d], Angus Gidley-Baird[a], John F Thompson[c,d] and Julian A Barden[a]

Extensive labelling for the apoptotic markers calcium channel receptor $P2X_7$ and caspase-3 and telomerase activity was co-localized at a similar intensity in areas affected by superficial spreading melanoma obtained from 80 patients. Labelling for each of these markers also extended 2μm from the melanoma into the keratinocyte layer of the adjacent normal epidermis. Conversely, the calcium-regulating receptors $P2X_{1-3}$ and $P2Y_2$ (found in normal but not neoplastic skin) were fully de-expressed within 2μm of the melanoma but fully expressed beyond that distance. The cell adhesion protein E-cadherin (also only present in normal skin) was progressively de-expressed from a point 2μm from the melanoma until full de-expression within the lesion. These results show that telomerase-induced proliferation and defensive apoptosis are co-localized and simultaneous processes in melanoma tissue. Melanoma cell proliferation appears to overwhelm the apoptotic defence, perhaps due to the anti-apoptotic effects of telomerase. In addition, keratinocyte regulation of the epidermis and dermis is severely compromised by the loss of E-cadherin and $P2X_{1-3}$ and $P2Y_2$ receptors, resulting in a lesion that is aggressive and malignant. *Melanoma Res* **13:137–145 © 2003 Lippincott Williams & Wilkins.**

Melanoma Research 2003, 13:137–145

Keywords: melanoma, purinergic receptors, malignancy, apoptosis

[a]Institute for Biomedical Research, Department of Anatomy and Histology, The University of Sydney, Sydney, NSW 2006, Australia, [b]Department of Anatomical Pathology, Royal Prince Alfred Hospital, Sydney, Australia, [c]The Melanoma and Skin Cancer Research Unit, The University of Sydney and Royal Prince Alfred Hospital, Sydney, Australia and [d]The Sydney Melanoma Unit, Royal Prince Alfred Hospital, Sydney, Australia.

Correspondence and requests for reprints to Dr Michael Slater, Institute for Biomedical Research, Department of Anatomy and Histology, The University of Sydney, Sydney, NSW 2006, Australia.
Tel: + 61 2 9351 5161 Fax: +61 2 9351 6556;
e-mail: michaels@anatomy.usyd.edu.au

Received 26 April 2002
Accepted 30 September 2002

Introduction

The incidence of malignant melanoma in many countries is increasing at a rate greater than that of any other cancer in humans [1]. Dysplastic naevi may be a precursor of melanoma in some cases. The early stages of melanoma are considered to be melanoma *in situ* and melanocytes in the radical growth phase (RGP). The advanced stages of the disease are primary melanoma in the vertical growth phase (VGP) and melanoma in the metastatic growth phase (MGP) [2].

The main defence against melanoma is programmed cell death (apoptosis), which is always involved in the spontaneous regression of neoplasms. Apoptosis also triggers the systemic immunological defences. Tumour treatments such as antineoplastic drugs, radiation and immunotherapy all include the initiation of apoptosis as part of their therapeutic action. Without apoptosis, tumour regression will not occur [3].

Most studies of melanoma have used melanotic cell lines, but the transformational biochemical changes that constitute early melanoma development are difficult to study in such models. Although VGP and MGP melanoma are available as cell lines, precursor lesions such as dysplastic naevi, melanoma *in situ* and RGP melanoma cannot be propagated *in vitro* [2].

In this study we used paraffin-embedded sections from 80 melanoma cases and 10 cases of normal skin to investigate apoptosis, P2X and P2Y receptor expression, telomerase activity, and E-cadherin cell adhesion protein changes in both normal skin and melanoma tissue. Serial sections were labelled using an amplified immunoperoxidase protocol. Expression of the apoptotic markers calcium channel receptor $P2X_7$ and caspase-3 or cysteine protease protein (CPP)-32, the ligand-gated purinergic calcium-receptor channels $P2X_{1-6}$, the metabotropic calcium receptor $P2Y_2$, the cell proliferation marker telomerase-associated protein, and the cell adhesion protein E-cadherin was studied in serial sections.

Materials and methods

A total of 80 cases of superficial spreading melanoma were examined. A small number of nodular and other histogenetic subtypes were also examined initially. Since the findings appeared to be similar in each of the various histogenetic subtypes tested, only cases of superficial spreading melanoma were chosen for the study, as this subtype best demonstrated both the depth and lateral extent of marker labelling.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections from previously diagnosed biopsies were used for this study.

0960-8931 © 2003 Lippincott Williams & Wilkins

DOI: 10.1097/01.cmr.0000056225.78713.42

Each section was immunolabelled as previously described [4]. Each slide was de-waxed in two changes of fresh Histoclear for 15 min each and then rehydrated. All sections were immersed in 3% hydrogen peroxide for 5 min, washed, and then incubated in the appropriate primary antibody (anti-P2X, anti-P2Y or anti-human telomerase-associated protein [hTP1]). The concentration of affinity-purified IgG used in each case was 1.25mg/ml in phosphate buffered saline (PBS) for 30 min. The slides were washed three times in PBS for 5 min each, followed by a 30 min incubation with secondary antibody from the LSAB kit (Dako). All slides were then again washed in PBS for 5 min, visualized using a 0.05% solution of diaminobenzidine (DAB) for 5 min, washed, dried and mounted in Entellan mounting medium (Merck). Approximately serial sections were stained with a standard haematoxylin and eosin (H&E) protocol. Pre-incubation with peptide epitopes at 5–10 μM completely blocked labelling in the pre-absorption controls.

The sections labelled with monoclonal anti-E-cadherin (Zymed, San Francisco, California, USA) were treated with primary antibody at a concentration of 1:100 in PBS for 30 min. Human P2X and P2Y subtype-specific antibodies were used as described elsewhere [5–8]. The antibody titres, defined as the reciprocal of the serum dilution resulting in an absorbance of 1.0 above background in the enzyme-linked immunosorbent assay (ELISA), were all in the range 75 000–95 000 compared with 200–250 for the pre-immune samples.

Production of hTP1 antiserum

The consensus sequence of hTP1 [9] was examined for suitable epitopes. A segment in the C-terminal domain corresponding to the segment Cys2524–Glu2540 was chosen and the peptide synthesized using standard tertiary butoxycarbonyl (t-BOC) chemistry on an ABI 430A synthesizer (Applied Biosystems, Foster City, California, USA) [10]. After high performance liquid chromatography (HPLC) purification, the peptide was cross-linked to diphtheria toxin using maleimidocaproyl-*N*-hydroxysuccinimide. The peptide–antigen conjugate was suspended in water at a concentration of 5 mg/ml and aliquots were emulsified by mixing with Complete Freund's Adjuvant. Emulsion volumes of 1 ml containing 2 mg of peptide epitope were injected intramuscularly into a sheep; second and subsequent boosts were given at intervals of 6 weeks using Incomplete Freund's Adjuvant. Blood samples were obtained by venepuncture after 12 weeks when adequate antibody titres had been reached. The blood was incubated at 37°C for 30 min, and stored at 4°C for 15 h. The serum was then collected following centrifugation and stored at −20°C in small aliquots. The serum samples were tested with an ELISA assay. The titre of antibody used in the experiments was 82 000 ± 3300 compared with 225 ± 25 for pre-immune serum. The antibody was affinity purified on Affi-Gel 10 (Biorad).

Results

Labelling for $P2X_7$, hTP1 and CPP32 was co-localized within the superficial spreading melanoma, while no labelling for these markers was detected in adjacent regions of normal skin in the melanoma samples or in the 10 separate sections of normal skin. In contrast, E-cadherin, $P2X_{1-3}$ and $P2Y_2$ were found in normal skin but not in melanoma.

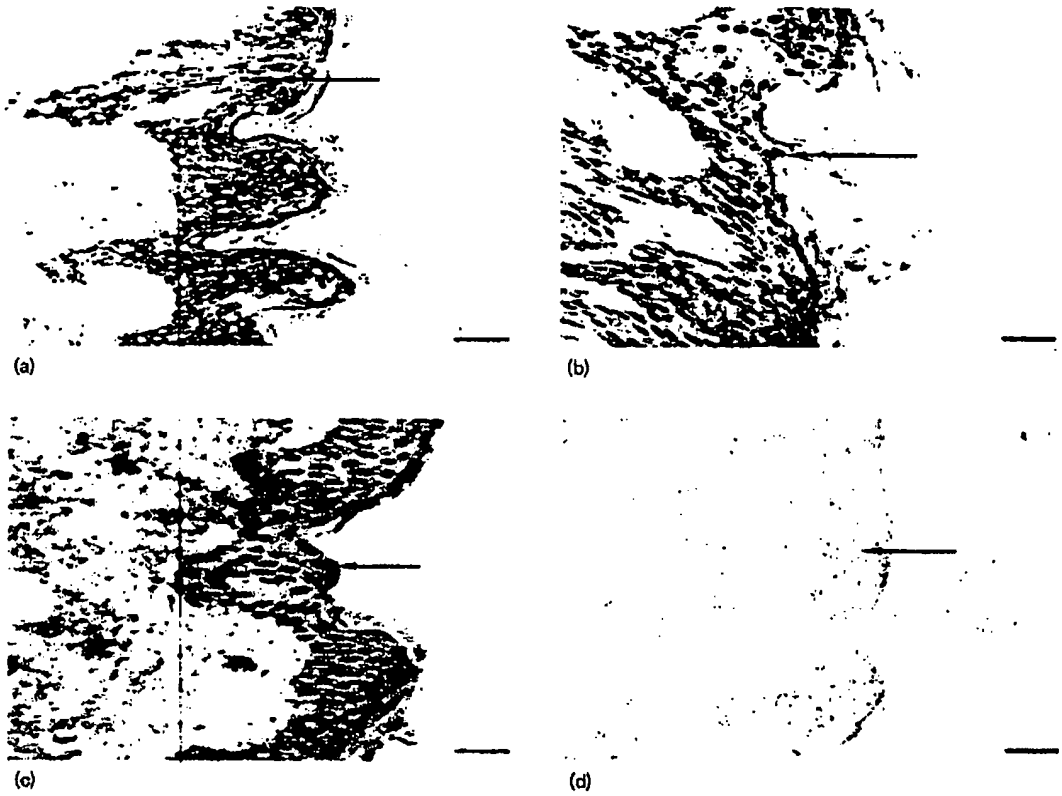
In the 10 normal skin cases and in the areas of normal skin not immediately adjacent to the melanomas, immunoperoxidase (IPX) labelling for the purinergic receptor $P2X_1$ took the form of distinct keratinocyte outlines (Fig. 1a). No label was observed in the stratum corneum. The bulk of the labelled cells were found in the stratum spinosum, with no particular concentration of labelled cells observed in the surrounding layers, either the stratum granulosum or the stratum basale. Strong nuclear labelling for $P2X_2$ (Fig. 1b) and $P2X_3$ (Fig. 1c) as well as the receptor $P2Y_2$ (results not shown) was also found throughout the stratum spinosum, with a sizeable proportion of all the epithelial cells in this layer exhibiting very similar intense labelling for all these receptors. $P2X_1$ labelling was relatively weak, with little apparent intracellular stain, indicating far lower protein expression than was evident for the other receptors. There was no detectable labelling for $P2X_{4-6}$ in either normal epithelium or in melanoma lesions (Fig. 1d). It was consistently evident that $P2X_2$, $P2X_3$ and $P2Y_2$ receptors were maximally expressed in normal skin further than 2 mm from the clear margins of a melanoma. An example of positive $P2Y_2$ labelling in normal epidermal cells is shown in Figure 2a. Within 2 mm of the clear margin of a melanotic lesion, however, all $P2X_{1-3}$ and $P2Y_2$ labelling was absent (Fig. 2b).

As the melanoma margins in these cases could be determined by H&E stain, it was possible to perform correlative microscopy and immunohistochemistry on approximately serial sections. Figure 3 shows H&E-stained sections of a well-defined melanoma (Fig. 3a) with normal epidermis on both borders (Fig. 3b). The arrow is a reference point 2 mm from any melanoma cells as defined by H&E labelling. Both sections show apparently normal epidermis at the edge of the melanoma.

IPX labelling for the cytolytic $P2X_7$ receptor (Fig. 4), the apoptotic marker CPP32 (Fig. 5a,b) and hTP1 (Fig. 5c,d) was co-localized and of similar intensity within the melanoma.

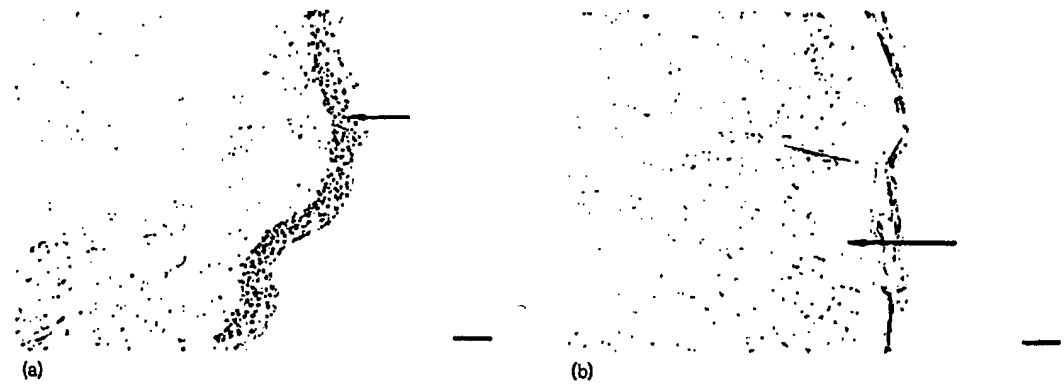
$P2X_7$, CPP32 and hTP1 were not labelled in normal

Fig. 1



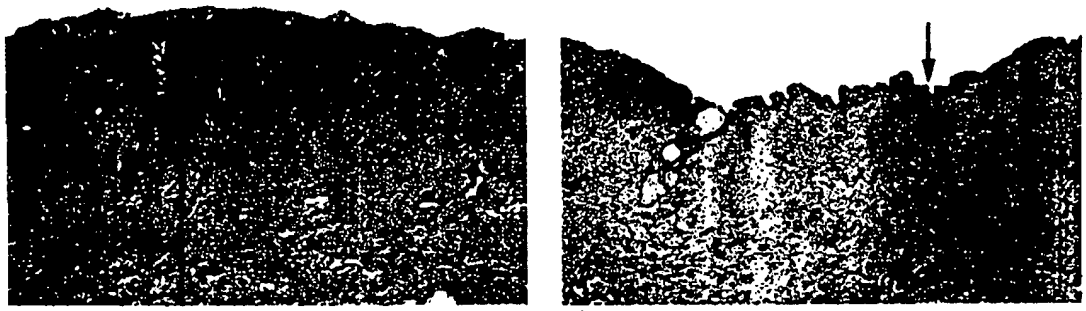
(a) IPX label for $P2X_1$ in normal epidermis. The label is in the form of nuclear outlines (arrow). (b) IPX label for $P2X_2$ in normal epidermis. An intense label occupies the nucleus of cells (arrow). (c) IPX label for $P2X_3$ in normal epidermis. The strong nuclear labelling pattern is very similar to that of $P2X_2$ (arrow). (d) IPX label for the P2X subtypes $P2X_{4-7}$ in normal epidermis. No label is present (arrow). Bar = 30 μm.

Fig. 2



(a) Low-power view of IPX labelling for $P2X_2$ in normal epidermis. Note that the label is positive and present all along the epidermis (arrow). (b) Within approximately 2 mm of melanoma cells, all labelling for $P2X_{2-3}$ disappears (arrow). Bar = 50 μm.

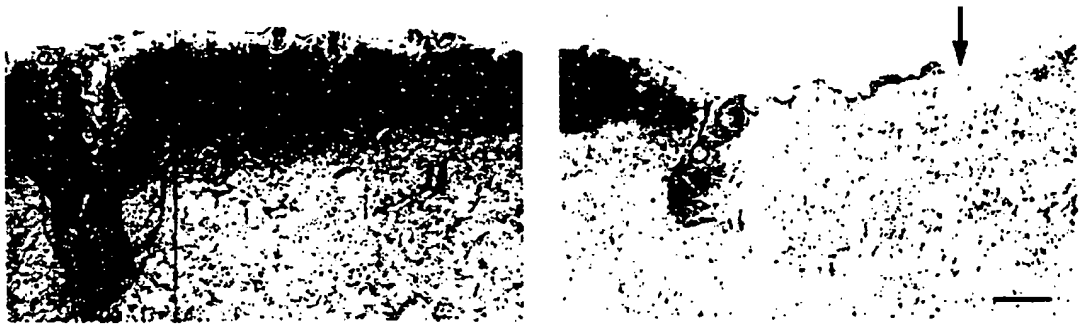
Fig. 3



(a) (b)

(a) H&E-stained section of an area of superficial spreading melanoma from the skin of the central back. (b) H&E-stained section of an area immediately adjacent to that shown in (a). About 75% of this micrograph shows normal skin. The point indicated by the arrow denotes the start of biochemically normal epidermis. No change is visible on H&E stain.

Fig. 4



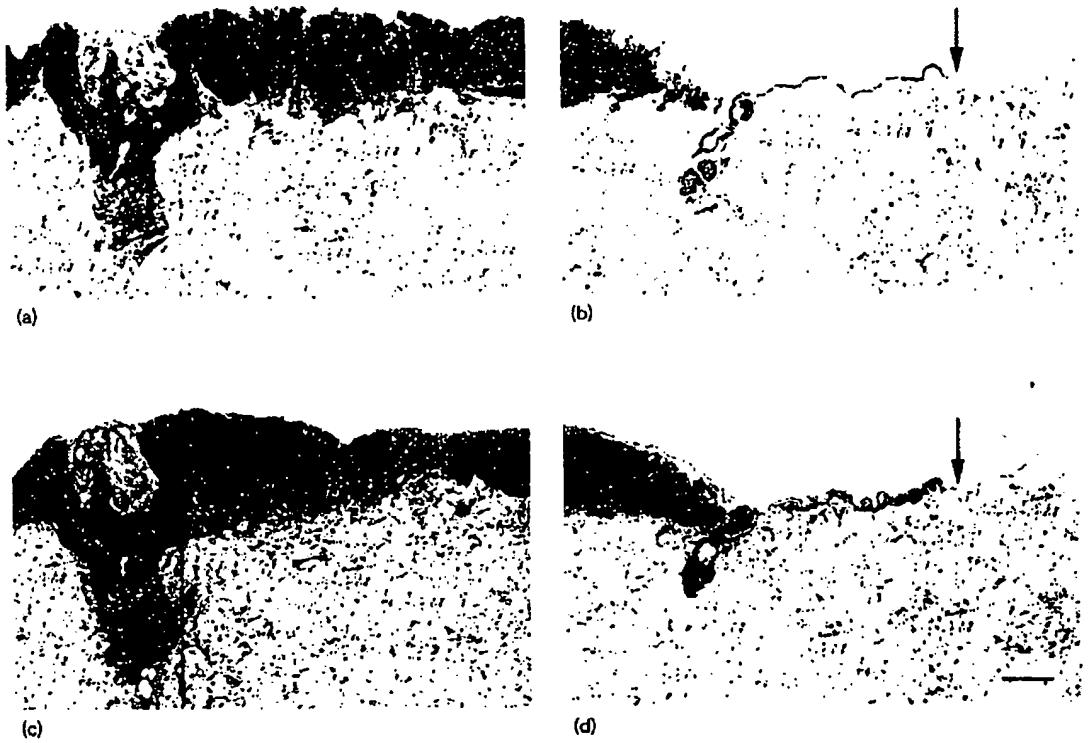
(a) (b)

(a) Approximately serial section showing IPX labelling for $P2X_7$. Expression of this cytolytic receptor/channel indicates that apoptosis is greatly upregulated in the melanoma. (b) Approximately serial section showing IPX labelling for $P2X_7$ in the area immediately adjacent to that shown in (a). Despite the apparently abrupt change between melanoma and normal skin as indicated by H&E stain (Fig. 3), some $P2X_7$ labelling extends past 2 mm from the melanoma into the adjacent, normal keratinocyte layer. From this point on (arrow), the skin does not show detectable $P2X_7$ expression and is probably biochemically normal. Bar = 0.5 μm.

skin in detectable amounts. In the melanoma tissue, as the labelling for $P2X_{1-3}$ disappeared within 2 mm of the tumour margins, the labelling for $P2X_7$, CPP32 and hTP1 progressively increased. Figure 4a shows labelling for $P2X_7$, indicating extensive expression of this receptor throughout the melanoma and extending out into the keratinocyte layer to a distance of approximately 2 mm (Fig. 4b). Figure 5a shows that the labelling of the apoptotic marker CPP32 correlates well with that of the $P2X_7$ receptors. There is an extensive and intense CPP32 label throughout the melanoma tissue that extends to the same reference point at 2 mm (Fig. 5b). Similarly, hTP1 is expressed with a similar intensity and distribution (Fig. 5c,d) as both $P2X_7$ and CPP32.

The cell adhesion protein E-cadherin was only expressed around keratinocytes. There was no detectable label for E-cadherin around normal melanocytes or melanoma cells. E-cadherin in the keratinocyte layer was de-expressed gradually with increasing proximity to a melanoma. Figure 6a is an H&E-stained section showing a well-defined melanoma with apparently normal adjacent skin that has been labelled with three reference points. There was no label for E-cadherin within the epidermis overlying the melanoma (Fig.

Fig. 5



(a) Approximately serial section showing labelling for CPP32. Expression of this apoptotic marker is a further indication that apoptosis is widespread in the melanoma. (b) Approximately serial section showing IPX CPP32 labelling in the area immediately adjacent to that shown in (a). As with $P2X_7$ expression, some of the CPP32 labelling in the keratinocytes extends up to 2 mm from the melanoma cells. From this point on (arrow), the skin does not show detectable CPP32 expression and is probably biochemically normal. (c) Approximately serial section showing labelling for hTP1. As with $P2X_7$ and CPP32 labelling in this study, telomerase activity is widespread in the melanocytic areas. (d) Approximately serial section from adjacent to (c) showing IPX hTP1 labelling. As with $P2X_7$ and CPP32, there is extensive telomerase activity within the melanoma. This label extends in the keratinocyte layer up to 2 mm from the melanoma. Beyond this point (arrow), the skin is probably biochemically normal. Bar = 0.5 μm.

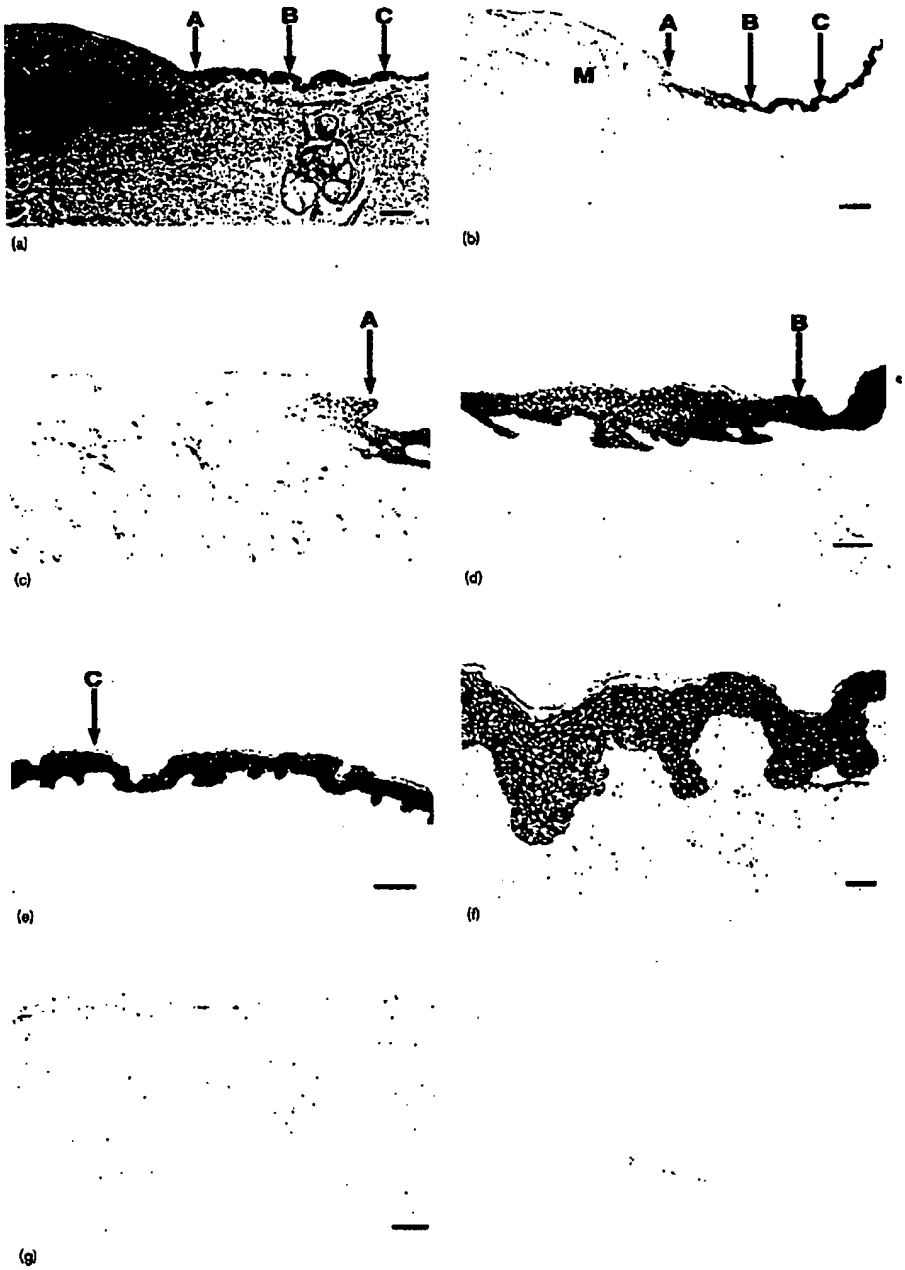
6a,b,c). However, immediately adjacent to the lesion some faint E-cadherin label was seen in the keratinocyte layer (Fig. 6c,d). The E-cadherin labelling increased to a moderate intensity approximately 1.2 mm from the lesion (Fig. 6b,d), but expression was not fully restored until a distance of ≥ 2 mm of the melanoma (Fig. 6e). Figure 6f is a high-power micrograph of an area of maximal epidermal E-cadherin expression, while Figure 6g demonstrates full de-expression of E-cadherin in the epidermis directly adjacent to a melanoma.

Discussion

In the current study it was clear that there was extensive, co-localized and intense apoptotic and telomerase activity in melanoma, as revealed by the expression of CPP32, the cytolytic $P2X_7$ receptor and hTP1.

Squamous epithelial cells (keratinocytes) are major sites for the biosynthesis of cytokines and growth factors. Keratinocytes are active cells with a 7 day turnover. They not only influence keratinization, but also the function of the underlying dermis. In turn, dermal cells release cytokines and proteases that regulate endothelial cells and keratinocytes. The coordinated function of multiple epidermal and dermal cell populations allows the skin immune system to respond rapidly and effectively to a wide variety of insults. Keratinocytes are the first line of defence in the skin immune system, and keratinocyte-derived cytokines are pivotal in mobilizing leukocytes from blood and for signalling other cutaneous cells. Cytokine-mediated cellular communication also enables dermal fibroblasts and endothelial cells lining the cutaneous vasculature to participate in immune and inflammatory responses [11]. In the current study, CPP32, $P2X_7$ and hTP1 were also expressed

Fig. 6



(a) H&E-stained section of another case in which normal skin is adjacent to a melanoma. Arrows A, B and C are reference points for parts (b) to (e). (b) IPX E-cadherin labelled detail of (a). This low power overview shows a complete de-expression of E-cadherin in most cells of the melanoma, with a faint (A), medium (B) and complete (C) progressive expression of E-cadherin occurring gradually until approximately 2 mm from the melanoma (C), from which point completely normal expression of E-cadherin in the keratinocytes of the epidermis is observed. Bar = 300 μm. (c) Medium-power micrograph of E-cadherin de-expression in the epidermis immediately adjacent to the melanoma. (d) Medium-power micrograph of partial E-cadherin expression at a distance of 1 mm from the melanoma (B). Bar = 100 μm. (e) Medium-power micrograph of normal E-cadherin expression from approximately 2 mm from the melanoma (C). Bar = 100 μm. (f) IPX E-cadherin labelled high-power micrograph of the labelling pattern of E-cadherin in the keratinocyte layer of the normal epidermis. Bar = 25 μm. (g) High-power micrograph of the labelling pattern of E-cadherin in the epidermis immediately adjacent to a melanoma. The label appears to be completely de-expressed. Bar = 25 μm.

in the keratinocyte layer. Each marker was expressed in the keratinocytes of apparently normal epithelium within 2 mm of a melanoma margin. Each was abruptly de-expressed at this distance from the melanoma margin, and none of these markers were expressed in the normal epithelium. This finding suggests that the biochemical changes associated with melanoma extend to a distance of 2 mm from the lesion.

Conversely, the cell adhesion protein E-cadherin was fully expressed on the plasma membrane of all keratinocytes in normal skin but was not expressed in normal melanocytes or melanoma cells to a detectable level. This finding suggests that melanocytes are more freely able to migrate than are keratinocytes, a factor that is important in malignancy. In the keratinocyte layer, E-cadherin was fully expressed beyond a distance of approximately 2 mm from the melanoma margin.

Cell–cell cross-talk mediated by cadherins and connexins results in coordinated regulation of cell growth, differentiation, apoptosis and migration. Abnormal expression of adhesion receptors and breakdown of intercellular communication appears to drive tumour development and progression [12]. In this study, loss of E-cadherin in the keratinocyte layer accompanied melanoma development. This frees melanocytic cells from the regulatory mechanisms of the keratinocyte layer, providing melanoma cells with an increased opportunity to migrate [13]. A key event in cancer metastasis is the transendothelial migration of tumour cells. Prior to migration, adhesion molecules are de-expressed at the endothelial junction, which is then penetrated by melanoma cell pseudopods. If the keratinocytes-dermal-endothelial cell regulation pathway is disrupted, as suggested by the current study, endothelial cells can assist the transmigration of tumour cells [14].

Like E-cadherin, the ligand-gated purinergic ionic calcium channels $P2X_{1-3}$ and the metabotropic calcium receptor $P2Y_2$ were expressed in the keratinocytes of normal skin but were abruptly de-expressed within a distance of approximately 2 mm from the melanoma. Intracellular calcium is known to be a potent regulator of apoptosis and androgen receptor expression [15]. The purinergic cytolytic calcium channel receptor $P2X_7$ is a major initiator of apoptosis in haemopoietic cells, as the activated receptor initiates the opening of a large pore in the plasma membrane. The resulting pore admits a large influx of ionic calcium, thus triggering apoptosis. The other P2X subtypes have differing characteristics. Each subtype modulates intracellular calcium in slightly different ways. The intracellular Ca^{2+} signal is transduced via more than 40 intracellular Ca^{2+}-binding proteins, including the S100 proteins, tenascin, calmodulin, integrin and annexin [16]. These calcium-bound proteins activate and regulate the cell cycle, protein secretion, composition of nuclear proteins, DNA transcription, apoptosis, adhesion protein binding, cell differentiation and phosphorylation [17]. Changes in cytosolic Ca^{2+} therefore control a wide range of cellular responses. Additionally, an increase in cytoplasmic calcium affects the binding of proteins to the cytoskeleton, induces the additional release of intracellular calcium [18], activates the Na^+/H^+ antiporter, increases cytoplasmic alkalinization [19], alters phosphatidylinositol metabolism [20] and activates protein kinases [21]. Calcium ions also trigger microtubule transport of membrane-bound organelles, exocytosis, and increases in nitric oxide synthase activity [22–26]. P2Y receptors increase intracellular Ca^{2+} levels by causing the release of Ca^{2+} from the non-mitochondrial, receptor-operated, intracellular calcium stores in the sarcoplasmic reticulum [27], whereas P2X receptors increase intracellular levels of Ca^{2+} by creating an ion-selective channel to the extracellular fluid [28]. Purines and pyrimidines can also either stimulate or inhibit proliferation depending on the extracellular microenvironment, the physiological state of the target cells, the cell cycle stage and the expression of P2 receptors [15].

Apoptosis can be initiated in a number of ways. Tumour necrosis factor (TNF) activates two distinct antiproliferative pathways including p38 mitogen-activated protein kinase (MAPK)-dependent cell cycle disruption and CPP32-mediated apoptosis [29]. Melanoma cell apoptosis is also regulated by endogenous nitric oxide resulting from inducible nitric oxide synthase activity [30]. The increase in intracellular calcium caused by proliferation of the $P2X_7$ receptors would not only initiate apoptosis, but also trigger increases in nitric oxide synthase activity [31], in turn inducing melanoma-specific apoptosis.

Telomerase labelling was a prominent feature of all the melanomas in the current study. Telomerase is a ribonucleoprotein enzyme that compensates for the progressive erosion of chromosomal ends, called telomeres. In most somatic cells telomerase expression is repressed and the telomeres progressively shorten after each cell division, causing cell senescence. Conversely, telomerase is active in most human cancers, maintaining the integrity of chromosome ends and representing an important step in cell proliferation, immortalization and carcinogenesis [32]. Telomerase plays a key role in carcinogenesis and is activated in most immortal cell lines and human cancers, including cutaneous melanoma [33]. Levels of telomerase activity and telomerase RNA in melanocytic lesions correlate well with clinical stage and could potentially assist in the diagnosis of borderline lesions [34]. The apparent lack of telomerase activity in a small percentage of reported cases is characteristic of the current antibodies that are based on human telomerase reverse transcriptase (hTERT)

epitopes. These appear to be easily masked or rendered conformationally unavailable. The antibody in the current study is based on a telomerase-associated protein, a more exposed epitope. In addition, a variable but significant percentage of apparently normal epidermis adjacent to a melanoma is positive for telomerase, indicating underlying biochemical cancer-associated changes that are not yet visible by H&E stain [32].

Links between telomerase activity, cell proliferation, apoptosis and expression of cell cycle regulators have not been extensively elucidated in cutaneous melanoma [33]. It has been proposed, however, that the regulatory mechanism controlling telomerase activity in melanoma relies on the transcription and alternative splicing of human telomerase reverse transcriptase [35]. In the current study, hTP1 and apoptosis markers were found in the same locations and with similar abundance. This probably indicates that apoptosis is very active as a defence against cancer cell proliferation, but that the neoplastic process overwhelms the apoptotic defence. There is also evidence that telomerase has an active anti-apoptotic role, as telomerase-expressing cells with elongated telomeres are resistant to apoptosis induced by hydroxyl radicals [36]. TNF also activates an anti-apoptotic mechanism in melanoma cells [29]. These findings may partly explain why apoptosis is ineffective as a defence against melanoma.

These results show that telomerase-induced proliferation and defensive apoptosis are co-localized and simultaneous processes in melanoma tissue. Melanoma cell proliferation appears to overwhelm the apoptotic defence, perhaps due to the anti-apoptotic effects of telomerase. In addition, keratinocyte regulation of the epidermis and dermis is severely compromised by the loss of E-cadherin and $P2X_{1-3}$ and $P2Y_2$ receptors, resulting in a lesion that is aggressive and malignant.

References

1 Lang PG Jr. Malignant melanoma. *Med Clin North Am* 1998; 82(6):1325–1358.
2 Yang P, Becker D. Telomerase activity and expression of apoptosis and anti-apoptosis regulators in the progression pathway of human melanoma. *Int J Oncol* 2000; 17(5):913–919.
3 Kaiser HE, Bodey B Jr, Siegel SE, Groger AM, Bodey B. Spontaneous neoplastic regression: the significance of apoptosis. *In Vivo* 2000; 14(6):773–788.
4 Slater M, Delprado WJ, Murphy CR, Barden JA. Detection of preneoplasia in histologically normal prostate biopsies. *Prostate Cancer Prostatic Dis* 2001; 4:92–98.
5 Moore K, Ray F, Barden J. Loss of expression of purinergic $P2X_3$ and $P2X_5$ receptors in adults with overactive detrusor contractility and urge incontinence. *J Neurosci* 2001; 21:U17–U22.
6 Ray F, Huang W, Slater M, Barden J. Purinergic receptor distribution in endothelial cells in blood vessels: a basis for selecting coronary artery grafts. *Atherosclerosis* 2002; 162:55–61.
7 Dutton J, Hansen M, Barden J, Bennett M. Development of the distribution of single P2X homomeric and heteromeric receptor clusters on smooth muscle cells in relation to nerve varicosities in the rat urinary bladder. *J Neurocytol* 1999; 28:3–15.
8 Barden J, Cottee L, Bennett M. Vesicle-associated proteins and P2X receptor clusters at single sympathetic varicosities in mouse vas deferens. *J Neurocytol* 1999; 28:469–480.
9 Harrington L, McPhail T, Mar V, Zhou W, Oulton R, Bass M, *et al.* A mammalian telomerase-associated protein. *Science* 1997; 275: 973–977.
10 Barden JA, Cuthbertson RM, Jia-Zhen W, Moseley JM, Kemp BE. Solution structure of PTHrP(Ala15)(1–34). *J Biol Chem* 1997; 272:29572–29578.
11 Williams IR, Kupper TS. Immunity at the surface: homeostatic mechanisms of the skin immune system. *Life Sci* 1996; 58(18):1485–1507.
12 Li G, Herlyn M. Dynamics of intercellular communication during melanoma development. *Mol Med Today* 2000; 6(4):163–169.
13 Li G, Satyamoorthy K, Herlyn M. N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. *Cancer Res* 2001; 61(9):3819–3825.
14 Voura EB, Sandig M, Siu CH. Cell-cell interactions during transendothelial migration of tumor cells. *Microsc Res Tech* 1998; 43(3):265–275.
15 Gong Y, Blok LJ, Perry JE, Lindzey JK, Tindall DJ. Calcium regulation of androgen receptor expression in the human prostate cancer cell line LNCaP. *Endocrinology* 1995; 136(5):2172–2178.
16 Heizmann C, Cox J. New perspectives on S100 proteins: a multifunctional Ca^{2+}-, Zn^{2+}- and Cu^{2+}-binding protein family. *Biometals* 1998; 11(4):383–397.
17 Keirsebilck A, Bonne S, Bruyneel E, Vermassen P, Lukanidin E, Mareel M, *et al.* E-cadherin and metastasin (Mts-1/S100a4) expression levels are inversely regulated in two tumor cell families. *Cancer Res* 1998; 58(20):4587–4591.
18 Jaconi ME, Theler JM, Schlegel W, Appel RD, Wright SD, Lew PD. Multiple elevations of cytosolic-free Ca^{2+} in human neutrophils: initiation by adherence receptors of the integrin family. *J Cell Biol* 1991; 112(6):1249–1257.
19 Schwartz M, Ingber D, Lawrence M, Springer T, Lechene C. Multiple integrins share the ability to induce elevation of intracellular pH. *Exp Cell Res* 1991; 195:533–535.
20 Cybulsky AV, Carbonetto S, Cyr MD, McTavish AJ, Huang Q. Extracellular matrix-stimulated phospholipase activation is mediated by beta 1-integrin. *Am J Physiol* 1993; 264(2 pt 1):C323–332.
21 Komberg L, Juliano R. Signal transduction from the extracellular matrix: the integrin-tyrosine kinase connection. *Trends Pharmacol Sci* 1992; 13:93–95.
22 Figueroa JP, Massmann GA. Estrogen increases nitric oxide synthase activity in the uterus of nonpregnant sheep. *Am J Obstet Gynecol* 1995; 173(5):1539–1545.
23 Pouli AE, Emmanouilidou E, Zhao C, Wasmeier C, Hutton JC, Rutter GA. Secretory-granule dynamics visualized in vivo with a phogrin green fluorescent protein chimaera. *Biochem J* 1998; 333(pt 1):193–199.
24 Chvatchko Y, Valera S, Aubry JP, Renno T, Buell G, Bonnefoy JY. The involvement of an ATP-gated ion channel, P2X1, in thymocyte apoptosis. *Immunity* 1996; 5(3):275–283.
25 Carabelli V, Carra I, Carbone E. Localized secretion of ATP and opioids revealed through single Ca^{2+} channel modulation in bovine chromaffin cells. *Neuron* 1996; 20(6):1255–1268.
26 Valera S, Talabot F, Evans RJ, Gos A, Antonarakis SE, Morris MA, *et al.* Characterization and chromosomal localization of a human P2X receptor from the urinary bladder. *Receptors Channels* 1995; 3(4):283–289.
27 Babich LG, Burdyga ThV, Shlykov SG, Kosterin SA. Evidence for Mg^{2+}, ATP-dependent accumulation of Ca^{2+} by the intracellular non-mitochondrial calcium store in uterine smooth muscle cells. *Ukr Biokhim Zh* 1997; 69(2):19–29.
28 Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A, *et al.* A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. *Nature* 1994; 371(6497):516–519.
29 Hattori T, Hayashi H, Chiba T, Onozaki K. Activation of two distinct anti-proliferative pathways, apoptosis and p38 MAP kinase-dependent cell cycle arrest, by tumor necrosis factor in human melanoma cell line A375. *Eur Cytokine Netw* 2001; 12(2):244–252.
30 Salvucci O, Carsana M, Bersani I, Tregni G, Anichini A. Antiapoptotic role of endogenous nitric oxide in human melanoma cells. *Cancer Res* 2001; 61(1):318–326.
31 Batra S, Al-Hijji J. Characterization of nitric oxide synthase activity in rabbit uterus and vagina: downregulation by estrogen. *Life Sci* 1998; 62(23):2093–2100.
32 Orlando C, Gelmini S, Selli C, Pazzagli M. Telomerase in urological malignancy. *J Urol* 2001; 166(2):666–673.
33 Miracco C, Pacenti L, Santopietro R, Biagioli M, Fimiani M, Perotti R, *et al.* Detection of telomerase activity and correlation with mitotic and apoptotic indices, Ki-67 and expression of cyclins D1 and A in cutaneous melanoma. *Int J Cancer* 2000; 88(3):411–416.

34 Ramirez RD, D'Atri S, Pagani E, Faraggiana T, Lacal PM, Taylor RS, *et al.* Progressive increase in telomerase activity from benign melanocytic conditions to malignant melanoma. *Neoplasia* 1999; 1(1):42–49.
35 Villa R, Porta CD, Folini M, Daidone MG, Zaffaroni N. Possible regulation of telomerase activity by transcription and alternative splicing of telomerase reverse transcriptase in human melanoma. *J Invest Dermatol* 2001; 116(6):867–873.
36 Ren JG, Xia HL, Tian YM, Just T, Cai GP, Dai YR. Expression of telomerase inhibits hydroxyl radical-induced apoptosis in normal telomerase negative human lung fibroblasts. *FEBS Lett* 2001; 488(3):133–138.